

**The Effect of Cytotoxic Chemotherapy on the Structural and Material
Properties of Regenerate Bone in a Rabbit Model of Limb Lengthening**

Fergal Monsell

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I, Fergal Monsell, confirm that the work presented in this thesis is my own. When information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

A New Zealand White (NZW) rabbit model was used to investigate the effect of cyclical cytotoxic chemotherapy on the structural and material properties of regenerate bone. This attempted to reproduce the biological situation encountered in a human adolescent with a primary malignant bone tumour, treated by surgical resection and either adjuvant or neo-adjuvant cyclical chemotherapy with bone transport to reconstruct the skeletal defect.

General Hypothesis

It is possible to produce normal bone by distraction osteogenesis in the presence of cyclical cytotoxic therapy.

Materials and Methods

Forty immature male rabbits were divided equally into 2 groups. Each received 2 cycles of either cis-platinum / adriamycin or normal saline, with a tibial osteotomy and lengthening at 12-weeks of age. The timing of the cytotoxic drugs differed between groups in an attempt to simulate an adjuvant and neo-adjuvant dose schedule.

Results

A reproducible animal model was developed, appropriate doses of cis-platinum and adriamycin were determined and it was demonstrated that surgical lengthening was possible in animals receiving chemotherapy.

There were no differences in the physical characteristics of the regenerate or lengthened bone in either arm of the study.

In the group that received 2-cycles of chemotherapy before lengthening (neo-adjuvant group), there was a significant reduction in bone mineral concentration (BMC), bone mineral density (BMD) and volumetric bone mineral density (vBMD), assessed by dual X-ray absorptiometry (DXA). There was no effect on the structural properties assessed by compression testing

In the group that received chemotherapy before and during lengthening (adjuvant group), there was no effect on mineralisation but a reduction in energy to yield and yield strain was demonstrated.

Conclusion

These findings should be interpreted with caution, as the animals did not have malignant bone tumours and were given a limited drug regimen. The study did not demonstrate any consistent effect on the properties of regenerate bone but the assessment did not include histological analysis. Further work is needed to investigate the mechanism in which these agents affect distraction osteogenesis and this will require a dynamic assessment of bone formation and more sophisticated analysis of regenerate structure.

For Ros, Ellie, Amy and Liam

Section 1 Osteogenic Sarcoma; Historical Perspectives, Current Concepts and the Aim of This Work

1.1 Introduction

My clinical interest in this subject is based on fifteen-years experience using distraction osteogenesis in the context of paediatric limb reconstruction. The motivation for this work is an ambition to investigate the use of this technique in the treatment of patients with osteogenic sarcoma.

This section presents an overview of the management of malignant tumours of the immature skeleton, concentrating on the development of pharmacological and surgical strategies that are in current use. An appreciation of the potential deficiencies in contemporary management is necessary to understand the process that led to the general hypothesis and experimental component of this thesis.

The description concentrates on osteogenic sarcoma as the index disease and defines the demographics and clinical features relevant to a Northern European population. Advances in the management over the last 4-decades are described, to highlight the very significant improvements in outcome that have occurred as a result of parallel contributions from disciplines including surgery, medical oncology and bioengineering.

Advances in cytotoxic chemotherapy are discussed with reference to complex drug combinations, which became prevalent in the 1980's and more recent 2-drug prescriptions, which form the basis of the experimental pillar of this work.

Adriamycin and cis-platinum are considered in detail, as it is necessary to understand how the pharmacology of these drugs in general and side effects in particular, influenced the development of the experimental model.

Contemporary surgical approaches are evaluated to highlight the advantages and potential limitations of each technique. A systematic review of the literature available at the time of writing considers the use of distraction osteogenesis in treatment of benign and malignant tumours.

The purpose of this review is to identify areas of technical difficulty and explore potential advantages of distraction osteogenesis in the management of osteogenic sarcoma.

1.2 Osteogenic sarcoma

Osteogenic sarcoma is the most common primary malignant tumour involving bone with approximately 1200 new cases diagnosed in European children each year. (Stiller et al., 2006) The age standardised rate for European patients up to 15-years is 2.8 cases per million and 6.8 cases per million between the age of 10 and 14-years. (Stiller et al., 2006)

Osteogenic sarcoma is a mesenchymal malignancy in which the malignant cell population produces a variable amount of osteoid, leading to a spectrum of clinical subtypes. Approximately 80% are high-grade central tumours, which are classified as osteoblastic, chondroblastic or fibroblastic. The typical presentation involves pain and swelling of the affected limb, most commonly the metaphyseal regions of the femur, tibia and proximal humerus.

Telangectatic, high-grade surface and small cell osteogenic sarcomas have a similar clinical course and treatment involves multi-modal chemotherapy.

There have been important advances in the management of malignant bone tumours in children over the last 4-decades. Improvements have occurred because of progress in many disciplines, but the development of potent cytotoxic drugs has been of fundamental importance. (Eilber et al., 1987, Jaffe et al., 1974, Link et al., 1986, Rosen et al., 1983, Rosen et al., 1976)

Local control is also essential and failure to remove the primary tumour completely is associated with a high risk of local and metastatic recurrence even with adjuvant chemotherapy. (Bacci et al., 1998a, Bacci et al., 1998b)

In 1966, McKenna *et al* (McKenna et al., 1966) reported the 5-year survival of a group of 552 patients with osteogenic sarcoma. Irrespective of whether they were treated or not treated and disregarding the mode of treatment, the 5-year survival rate was 22.7% and long-term survival rate was 24.5% after amputation and 20.5% after resection.

In 1967, Dahlin *et al* (Dahlin and Coventry, 1967) reported the outcome of 410 patients with osteogenic sarcoma and noted an overall 5-year survival of 20.3% and 25.4% for patients with humeral and femoral tumours.

In 1973, Sweetnam (Sweetnam, 1973) reported a 5-year survival of 26% for patients with distal femoral osteogenic sarcoma, treated by high thigh amputation or hip disarticulation. Pulmonary metastases developed in a large proportion of these patients, indicating that control of metastatic disease was a major factor in determining survival.

1.3 Multi-agent Chemotherapy in the Management of Osteogenic Sarcoma

The introduction of multi-modal chemotherapy in the early 1970's produced significant improvements in the treatment of patients with metastatic osteogenic sarcomas. Complex treatment protocols evolved which are broadly defined as adjuvant or neo-adjuvant. Adjuvant chemotherapy involves the administration of chemotherapeutic agents following surgery. Neo-adjuvant chemotherapy refers to chemotherapy that is employed prior to surgical treatment.

The use of multi-agent chemotherapy was first reported in the 1980's. (Eilber *et al.*, 1987, Link *et al.*, 1986, Rosen, 1985, Rosen *et al.*, 1983) Rosen (Rosen, 1985) reported the 10-year results of 208 patients with primary osteogenic sarcomas of the extremity, treated with pre-operative chemotherapy using one of 4 treatment protocols.

At a minimum follow-up interval of 36-months, 77% of 87 patients treated on the Memorial Sloan-Kettering Cancer Centre T10 regimen remained alive and continuously disease free and 81.6% were free of disease at most recent assessment. This protocol involved pre-operative high dose methotrexate and leucovorin with surgery on day-28. High dose methotrexate, leucovorin, bleomycin, cyclophosphamide, actinomycin and adriamycin were administered in combination following surgery.

The Multi Institutional Osteosarcoma Study (MIOS) was initiated in June 1982 and used high dose methotrexate, adriamycin, cis-platinum, bleomycin, cyclophosphamide and actinomycin D. Patients were randomly assigned to

immediate adjuvant chemotherapy or to observation without adjuvant treatment after definitive surgical management of the primary tumour. The study consisted of 113 eligible patients of whom 36 accepted the randomisation and 18 were assigned to immediate adjuvant chemotherapy and 18 to observation.

The initial results were reported in 1986 (Link et al., 1986) and midterm results in 1991 (Link et al., 1991). The 6-year disease free survival rate for the control group was 11% compared to 61% for patients receiving chemotherapy. Patients who declined randomisation but were treated according to protocol also demonstrated a significant survival advantage.

Eilber *et al* (Eilber et al., 1987) reported a study involving 59 patients with non-metastatic, classic intra-medullary osteogenic sarcoma. Thirty-two were randomised to receive high dose methotrexate, adriamycin, bleomycin, cytoxan, and actinomycin D, 27 did not receive adjuvant chemotherapy. At a mean follow-up of 2-years, there was a statistically significant improvement in disease-free and overall survival in those who received adjuvant chemotherapy.

1.3.1 cis-Platinum and Adriamycin in the Treatment of Osteogenic Sarcoma

In 1981, Ettinger *et al* (Ettinger et al., 1981) reported the use of cis-platinum and adriamycin following definitive surgery in 12 patients with non-metastatic primary osteogenic sarcoma. All patients were alive and 10 remained continually disease free at a median time of 23-months.

In 1991, Malawer *et al* (Malawer et al., 1991) used 2 cycles of pre-operative, intra-arterial cis-platinum and intravenous adriamycin for high-grade sarcomas of the extremity. This group of patients presented with osteogenic sarcoma, malignant fibrous histiocytoma, leiomyosarcoma and malignant schwannoma. All patients received post-operative cycles of cis-platinum and adriamycin and at median follow-up of 30-months, 95% of patients (21/22) had evidence of local control. Six (27.3%) however, developed metastatic disease at a median interval of 16.6-months.

The European Osteosarcoma Intergroup (EOI) recruited 198 eligible patients between July 1983 and December 1986. These patients had classic, high-grade

extremity osteogenic sarcomas and were randomised to receive cis-platinum and adriamycin prior to and following definitive surgery or the same combination with high dose methotrexate.

Bramwell (Bramwell et al., 1992) reported the initial results at a mean time of 53-months and the disease free survival was superior in the cis-platinum and adriamycin group, although this was not statistically significant. The conclusion of this paper was that a brief, intensive regimen of cis-platinum and adriamycin produced excellent long-term results, which were equivalent to those using longer, more complex multi-agent combinations.

In 1997, Souhami *et al* (Souhami et al., 1997) reported the results of an EOI study that compared a short intensive treatment using cis-platinum and adriamycin with a multi-drug regimen, based on the T10 protocol. This involved pre-operative vincristine, high dose methotrexate and adriamycin and post-operative bleomycin, cyclophosphamide, dactinomycin, vincristine, methotrexate, adriamycin and cis-platinum.

Four hundred and seven patients were randomised, 390 were eligible and were followed up for at least 4-years. Surgery involved amputation or limb sparing surgery and this was determined on the basis of clinical preference and not trial randomisation. The overall survival was 65% at 3-years and 55% at 5-years in both groups. The authors observed that the 5-year survival was unsatisfactory and that new approaches including dose intensification were required.

1.3.2 cis-Platinum Pharmacology

cis-Platinum was first described in 1845 as Peyrone's salt. (Peyrone, 1845) Werner elucidated the molecular structure in 1893 and this formed part of the work that was recognised with the Nobel Prize for Chemistry in 1913. (Figure i)

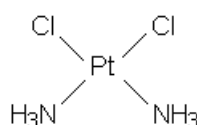


Figure i The Molecular Structure of cis-Platinum

The cytotoxic properties of cis-platinum were discovered by Rosenberg, (Rosenberg et al., 1965) who observed that electrolysis products from a platinum electrode inhibited mitosis in *Escherichia coli*.

Initial animal studies were conducted in 1971, (Kociba and Sleight, 1971) leading to a clinical trial involving 26 patients with a variety of malignancies in 1973 (Lippman et al., 1973) and approval for clinical use by the American Food and Drug Administration (FDA) in 1978.

cis-Platinum has been demonstrated to have an anti-neoplastic activity with a clinically useful anti-cancer effect on ovarian and testicular tumours, malignant melanoma and osteogenic sarcomas. Efficacy is limited by undesirable dose limiting side effects, which include severe nephrotoxicity, nausea, vomiting, neurotoxicity, ototoxicity and myelosuppression.

Cytotoxicity is mediated by inhibition of Deoxyribonucleic Acid (DNA) synthesis and this occurs at significantly lower doses than are required to inhibit Ribonucleic Acid (RNA) and protein synthesis. (Harder and Rosenberg, 1970) The mechanism of cis-platinum damage to DNA is similar to alkylating agents and X-irradiation (Weiss and Poster, 1982), binding to 2 specific sites on the DNA molecule. (Munchausen and Rahn, 1975) This causes inter-and intra-strand DNA binding in addition to DNA/protein cross linkage. (Javadpour, 1985) cis-Platinum appears to have a preferential toxicity to the DNA of cancer cells compared to normal cells, although this phenomenon is not completely understood. (Javadpour, 1985) The cross-linked DNA prevents rapidly dividing cells from duplicating DNA for mitosis. The damaged DNA triggers repair mechanisms, which lead to cell death by apoptosis when repair proves impossible.

1.3.3 Side Effects of cis-Platinum

Nephrotoxicity is common due to the fact that the kidney accumulates and retains platinum and is the primary method of excretion of platinum compounds. Toxicity results in renal tubular damage that may be reduced by vigorous hydration and mannitol induced diuresis. (Hayes et al., 1977)

The mechanism of nephrotoxicity induced by platinum complexes is not completely understood. Reports implicate site specific injury to the S3 segment (*pars recta*) of the proximal convoluted tubules (PCT) (Dobyan et al., 1980) and reaction of platinum complexes with protein bound sulphydryl groups present in the proximal tubules. (Appleton T et al., 1989) Other authors have reported the distal tubules (Gonzales-Vitale et al., 1977) and proximal and distal tubules (Jones et al., 1980) as the site of maximum damage.

1.3.4 Adriamycin Pharmacology

Adriamycin is an anthracycline antibiotic, produced by solvent extraction of the products of aerobic fermentation of *Streptomyces peucetius*. The molecular structure was described by Arcamone in 1969 (Arcamone et al., 1969) (Figure ii) and clinical trials began in 1970. (Middleman et al., 1971, Wiernik and Serpick, 1972) Adriamycin has subsequently been used as an anti-cancer agent to treat a spectrum of solid tumors and leukaemias. (Chabner et al., 1975)

Adriamycin acts by binding to DNA where it inhibits the enzyme topoisomerase II. Adriamycin stabilises the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being resealed and thereby stopping the process of replication. (Tewey et al., 1984)

Topoisomerase II is now known to be a common target for many families of DNA intercalating anti-cancer drugs. Other potential mechanisms of action include enzyme catalysed and ion mediated free radical formation (Handa and Sato, 1975) covalent binding to DNA, (Sinha et al., 1987) and direct interaction with the cell membrane. (Tritton, 1991)

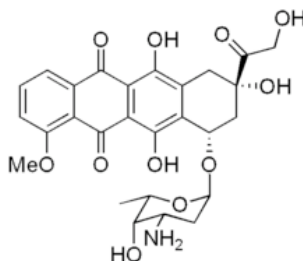


Figure ii The Molecular Structure of Adriamycin

1.3.5 Side Effects of Adriamycin

Clinical use is limited because of acute bone marrow toxicity, which has been partially improved with the use of bone marrow stem cell colony stimulating factors. The frequency of administration is limited because of drug resistance and side effects which include stomatitis, alopecia (Wang et al., 1971) and a delayed dose dependant cardiotoxicity, eventually resulting in congestive cardiac failure. (Billingham et al., 1978, Lenaz and Page, 1976, Minow et al., 1977, Von Hoff et al., 1979, Wang et al., 1971)

The exact mechanism that leads to cardiomyopathy is unknown, but oxygen free radical generation with subsequent lipid peroxidation, local release of vasoactive substances, calcium overload, mitochondrial dysfunction, and intercalation of adriamycin into DNA base pairs, leading to faulty cellular repair have all been implicated. (Bristow et al., 1981, Keizer et al., 1990, Lazarus et al., 1980, Miwa et al., 1986, Singal and Pierce, 1986, Zahringer, 1981)

Sakski *et al* (Sasaki et al., 1987) investigated the effect of adriamycin on collagen production and fibroblast proliferation in normal human skin fibroblast culture using [³H] hydroxyproline as a marker of collagen turnover. There was inhibition of fibroblast proliferation, generalised inhibition of protein synthesis and specific inhibition of prolyl hydroxylation leading to loss of stability of procollagen in the triple helix which may explain impairment of wound healing in patients treated with adriamycin

1.3.6 Overview of the Role of Chemotherapy in the Management of Osteogenic Sarcoma

Since the introduction of multi-modal chemotherapy in the 1980's, cis-platinum and adriamycin have been used successfully in the management of osteogenic sarcoma and Ewing's sarcoma.

Initial studies used complex drug prescriptions and produced a significant improvement in disease free survival. More recent studies demonstrated that an equivalent therapeutic effect could be achieved using a combination of cis-platinum and adriamycin.

The therapeutic use of these agents is limited due to significant side effects. Cis-platinum is a potent nephrotoxic agent and adriamycin produces cardiac and bone marrow toxicity, which also limits the use in a clinical context.

1.4 Surgical Management of Osteogenic Sarcoma in Childhood

Significant improvements in survivorship, independent of the primary method of treatment, led to advances in surgical reconstruction following osteogenic sarcoma excision. This section evaluates the development of these techniques and describes bone transport by distraction osteogenesis and its potential use in this context.

1.4.1 Historical Perspective

In 1986, Simon *et al* (Simon et al., 1986) conducted a retrospective, multi-institutional study of patients with osteogenic sarcoma of the distal femur. Three patient groups were identified who had either a limb sparing procedure, above knee amputation or disarticulation of the hip. There was no demonstrable difference in disease free interval or long-term survival between these groups.

The improvement in disease control associated with cytotoxic chemotherapy has resulted in patients presenting with conventional intra-medullary osteogenic sarcoma, without metastatic disease, expecting a cure rate of the order of 70%. Improved survival following chemotherapy, with equivalent results for amputation and limb salvage, (Eilber et al., 1987, Marcove, 1978, Springfield et al., 1988) led to advances in limb sparing surgery using biological and bioengineering techniques. Local control is a prerequisite for limb salvage and is achieved by surgical excision of the tumour with adjuvant or neo-adjuvant chemotherapy to control micro-metastatic disease.

Limb sparing procedures are possible when pre-operative staging demonstrates adequate surgical margins. This is based on the pathological examination of the surgical specimen and the presence of an inadequate surgical margin should lead to amputation, particularly if there had been a poor response to pre-operative chemotherapy. (Bacci et al., 1998a, Bacci et al., 1998b)

The aim of limb sparing surgery is to achieve complete excision of the tumour, followed by reconstruction of the defect with minimal morbidity and optimum function. The ideal operation involves complete excision of isolated, non-functional bones and this is appropriate for tumours affecting the ribs, fibula and lower scapula.

The options for biological reconstruction of long bones include allograft, vascularised free fibular graft and bone transport. The alternative is to use an implantable prosthetic device with or without the potential for longitudinal extension to accommodate for remaining growth.

Grimer *et al* (Grimer et al., 1997) investigated the cost implications of limb salvage and compared this to the estimated cost of an amputation including lifelong prosthetic requirements. This demonstrated financial advantages associated with endoprosthetic replacement compared with amputation either in the public or private sector.

1.4.2 Allograft Reconstruction

An allograft or allogeneic transplant uses tissue derived from a genetically non-identical member of the same species. The use of allograft in the treatment of patients with segmental bone loss that has occurred because of tumour or trauma has its roots in antiquity. The earliest contemporary study involved transplantation of the knee and was reported in 1925 (Lexer, 1925) with approximately 50% of the grafts demonstrating acceptable long term function.

Mankin *et al* (Mankin et al., 1987) reported the experience with 314 patients at the Massachusetts General Hospital between 1971 and 1986. Patients with follow-up of greater than 2-years were considered separately and accounted for 74% of the total. In this group, 77% were regarded as satisfactory but the rate of significant complications was high. The complications included death (7%), metastases (9%), local recurrence (7%), infection (14%), fracture (19%) and non-union (13%).

Gebhardt *et al* (Gebhardt et al., 1991) reported the results of 53 patients aged 30-years or younger in whom fresh frozen allografts were used as osteoarticular grafts, allografts, arthrodeses, allograft prosthesis or inter-calary grafts. Function

was satisfactory in 70% of 38 patients with more than 2-years follow-up with no evidence of local recurrence. Complications were also common and included infection (30%), fracture (11%), non-union (23%) and joint instability (11%).

The authors concluded that this was appropriate management for patients with high-grade osteogenic sarcoma who received aggressive adjuvant chemotherapy. The results were considered to be comparable to other methods of reconstruction and compared to metallic prostheses, offered the advantage of potential integration with added longevity.

Enneking *et al* (Enneking and Mindell, 1991) reported the radiographic and histological details of 16 retrieved massive allografts that had been implanted for between 4 to 65-months. Union between allograft and host occurred slowly, with formation of an external callus derived from the host cortex. Internal repair also occurred slowly and was confined to the superficial ends of the graft and only involved 20% of the graft at 5-years. There was no histological evidence of chondrocyte survival, even when the graft had been cryoprotected before freezing. Necrotic cartilage appeared to function well for as long as 5-years and was replaced by a pannus of fibro-vascular tissue.

Alman *et al* (Alman et al., 1995) reported a retrospective analysis of allograft reconstruction in 26 skeletally immature patients. The results were considered to be good in 70% of patients but there was a high rate of complications with fracture occurring in 54%. Significant limb length discrepancies were identified and 5 of this group were managed with contra-lateral epiphysiodesis and one with an unsuccessful attempt at limb lengthening. The authors considered that this was a useful option for the management of skeletally immature individuals, accepting that its use in the lower extremity should be easier for patients in whom leg length inequality could be treated with epiphysiodesis.

Donati *et al* (Donati et al., 2000) reported the results of 112 allografts to treat high-grade osteogenic sarcomas following neo-adjuvant chemotherapy. The allografts were used as an arthrodesis in 44 cases and inter-calary graft in 39. The functional results were excellent/good in 74% of patients after primary surgery and 83% after secondary surgery. There was a high complication rate with delayed

union in 49% and fracture in 27% but deep infection was not reported. It was felt that the delay in union was increased by the use of chemotherapy.

1.4.3 Vascularised Fibular Graft Reconstruction

In 1975, Taylor *et al* (Taylor et al., 1975) reported the first successful use of a vascularised fibular graft to reconstruct a tibial defect. The first description of use after bone tumour resection was by Weiland in 1981. (Weiland, 1981)

In 1997, Hsu *et al* (Hsu et al., 1997) reported 30 patients following free vascularised fibular transfer to reconstruct massive defects after resection of primary bone tumours. The graft was fixed with a plate in 18 patients, an intramedullary nail in 5 and an external fixator in 7 patients. All patients had supplementary iliac crest bone grafting with a union rate of 90% at an average of 7.6-months. The conclusion was that this was an effective method of reconstruction, although there was a high rate of complications (50%), which included infection and non-union and often required further surgery.

Shea *et al* (Shea et al., 1997) reported 13 consecutive patients with an average follow-up of 53-months. The average time to union was 6.5-months, significant graft hypertrophy occurred in 61% and the complication rate was 54% with graft removal in 15%. The average Musculoskeletal Tumour Society Score (MTSS) was 90, with 11 of the 13 patients rated good or excellent. The authors considered that this technique represented a potential option for reconstruction of skeletal defects after tumour resection.

Shalaby *et al* (Shalaby et al., 2006) reported 6 patients with primary osteogenic sarcoma of the distal tibia treated with en bloc resection and tibio-talar arthrodesis. The defect was reconstructed with a non-vascularised fibular graft (3) and a vascularised pedicle fibular graft (3). This was supplemented with iliac crest bone graft and an Ilizarov fixator was used to bridge the defect in all cases. All tumours were graded as Enneking stage II B and underwent 3 cycles of cis-platinum and adriamycin. The mean age of the patients was 18.8-years (16-23) and the mean follow-up time was 35-months (28-42). Two patients required secondary iliac crest bone grafting to achieve union with a mean time to graft union of 10-months (8-12) in patients with a vascularised graft and 18-months (16-22) in non-vascularised

grafts. All patients demonstrated evidence of graft hypertrophy at the time of follow-up apart from one patient with local recurrence, who required an above knee amputation.

1.4.4 Rotationplasty

Van Nes initially described rotationplasty in 3 patients with congenital femoral deficiency (van Nes, 1950) and reported excellent results in all patients after 8 to 10-years.

Kotz *et al* (Kotz and Salzer, 1982) reported rotationplasty for childhood osteogenic sarcoma in 4 patients, combined with an en-bloc excision of the proximal tibia, distal femur and knee. There was no clinical or radiological evidence of local recurrence at follow-up between 27 and 58-months following rotationplasty but one of this group had died of widespread metastatic disease.

Jacobs (Jacobs, 1984) described the results of the van Nes rotationplasty in 2 patients with osteogenic sarcoma. Both patients adapted well and enjoyed good postoperative function. The procedure was therefore considered a potential alternative to a high above knee amputation in a young patient with significant growth remaining.

Hillmann *et al* (Hillmann et al., 1999) compared the results of rotationplasty in 33 patients with endoprosthesis replacement in 34 patients, followed up for an average of 6-years. The groups were evaluated by questionnaire with functional results assessed by the MTSS. This study suggested that rotationplasty was not associated with any disadvantage in respect of function or quality of life. Restriction in daily activity due to pain was significantly less common in the rotationplasty group but there were significant cosmetic issues.

Hanlon *et al* (Hanlon and Krajchich, 1999) reported the results of 21 patients who underwent a modified van Nes rotationplasty for osteogenic sarcoma. Of this group, 14 were followed-up at a mean of 8-years and all had good or excellent results with no long-term effect on recreational, sporting or occupational goals.

1.4.5 Endoprosthetic Reconstruction

This approach was pioneered in the United Kingdom at the Royal National Orthopaedic Hospital (RNOH) in 1949 (Seddon and Scales, 1949) and the long-term follow-up was reported in 1975. (Burrows et al., 1975)

Roberts *et al* (Roberts et al., 1991) reported the experience from the Royal Orthopaedic Hospital in Birmingham with 135 custom made distal femoral prostheses. Survivorship analysis demonstrated a success rate of 72% at 5-years, 64% at 7-years and 91% of surviving patients reported good or excellent function. The conclusion was the prosthetic replacement of the distal femur could predictably provide good functional results with no more risk to life than would be expected following amputation.

Kabukcuoglu *et al* (Kabukcuoglu et al., 1999) reported endoprosthetic replacement of the proximal femur in 54 patients with a mean follow-up of 9-years. The survivorship of the prosthesis without revision was 77% at 10-years, and 57% at 20-years.

Torbet *et al* (Torbert et al., 2005) reported the results of a retrospective study of 139 endoprosthetic procedures of which, 110 were used to reconstruct the femur or tibia. The overall event free endoprosthetic survival was 86% at 3-years, 80% at 5-years and 69% at 10-year follow-up. Survival was not affected by patient age or whether the prosthesis was a primary or revision procedure. The rate of local recurrence was 6.8%, which was similar to previous limb salvage and amputation studies.

1.4.6 Expandable Endoprosthetic Reconstruction

Modular, extendable endoprosthetic replacements have been developed to accommodate future growth in younger patients. Most series involve small numbers with high rates of aseptic loosening, infection and prosthetic failure.

Eckardt *et al* (Eckardt et al., 1993) reported the outcome of 12 skeletally immature patients with cemented, customised expandable endoprosthesis with mean follow-up of 3.5-years for surviving patients. Eight patients had a total of 10 major

complications of which 7 were related to the prosthesis. The MTSS rating was good to excellent in 7 patients (58%), fair in 3 (25%) and poor in 2 (17%).

Schiller *et al* (Schiller et al., 1995) reported the results of 6 skeletally immature patients who had been managed with an expandable tumour endoprosthesis at a mean age of 11-years with an average follow-up of 6-years. The mean length gain was 13.1 cm but this required a total of 53 procedures including 7 revisions. All cases demonstrated a good or excellent functional result with no local recurrences or evidence of metastatic disease at follow-up. Three of these patients developed deep infection, which required debridement, immediate re-implantation of the endoprosthesis and long-term antibiotics.

Schindler *et al* (Schindler et al., 1997) reported the experience from the RNOH between 1983 and 1990. Eighteen children were treated with an extendable distal femoral prosthesis, 4 had died from metastatic disease, 2 required amputation and 12 were followed for a mean time of 8.7-years. In the follow-up group, a mean total lengthening of 5.2 cm was achieved after an average of 4 operations. Six patients required revision at a mean of 6.2-years after the initial procedure and at final review, the average function assessed by the MTSS score was 77% of the expected normal function.

Gupta *et al* (Gupta et al., 2006) reported the early experience of the use of a non-invasive distal femoral expandable endoprosthesis in 7 patients with osteogenic sarcoma of the distal femur at a mean age of 12.1-years. The prostheses were lengthened by a mean of 25 mm (4.25-55) and the MTSS was 68 at mean follow-up of 20.2-months. Reported complications included a knee flexion deformity of 25° in one patient and death from disseminated disease in one patient.

1.4.7 Overview of the Surgical Management of Osteogenic Sarcoma

Surgical treatment of osteogenic sarcoma has evolved from amputation 4-decades ago to contemporary methods of biological and prosthetic reconstruction. There are advocates for each approach, but the literature suggests that there are major complications associated with all currently available methods of reconstruction.

Allograft reconstruction is associated with a significant rate of infection and fracture at the allograft-bone interface and the use of alternatives including vascularised fibular grafts and rotationplasty has not been extensive.

Recent advances in biomaterials and engineering have led to fixed and expandable endoprosthetic reconstruction. This is relevant to reconstruction in a child before skeletal maturity, which is the clinical scenario considered by this thesis. The literature reporting the short to mid-term use of these devices, suggests that there is also a high rate of infection and implant failure and there is no data relating to long-term use.

1.5 Distraction Osteogenesis

This section describes the origins of the technique of distraction osteogenesis and evaluates its use in the management of bone loss and deformity correction.

Recent refinements of technique have broadened the indications to include reconstruction following excision of benign and malignant tumours and these are also discussed.

1.5.1 Historical Perspective

Ilizarov used the term “regenerate” to describe the bone formed by distraction osteogenesis and investigated the biological and mechanical factors that influenced bone formation. (Ilizarov, 1989a, Ilizarov, 1989b, Ilizarov, 1990, Ilizarov and Ledyayev, 1992) The conclusion of these experiments, in addition to 40-years clinical experience, was that osteogenic activity depended on the degree of stability of the external fixator and the amount of damage to the bone marrow, periosteum and intramedullary nutrient vessels occurring at the time of surgery.

Ilizarov also published experimental evidence outlining the effect of the rate and frequency of distraction (Ilizarov, 1989a, Ilizarov, 1989b) and demonstrated that 1mm/day in 60 increments was superior to 4 or one increments. This study also demonstrated that the soft tissues (fascia, nerves and vessels) lengthened at this rate and rhythm, replicated and took on the characteristics of foetal tissue.

De Bastiani *et al* popularised distraction osteogenesis in Europe with the Orthofix mono-lateral external fixator and introduced the term ‘callotaxis’. (De Bastiani *et al.*, 1987) The technique involved a tissue preserving osteotomy and after a delay,

distraction at 1mm/day. When the target length was achieved, the fixator was dynamised, allowing load sharing between fixator and bone. The results of 100 lengthening procedures were reported with an average increase in length of 6 cm with a mean increase of 22% of bone length. Complications occurred in 14% of which 5% involved refracture.

1.5.2 The Biology of Distraction Osteogenesis

Aronson *et al* (Aronson et al., 1989) investigated the biology of distraction osteogenesis and used the Ilizarov and Wagner external fixators to lengthen the tibia by 2.6 mm in 2 groups of adult dogs with the contralateral tibia acting as a control. Eight were lengthened with a basic Ilizarov device, with 2 rings and 4 tensioned 1.6 mm pins and 8 with the small Wagner external fixator.

The tibiae were analysed with standard radiographs at intervals during the osteogenic process and it was observed that bridging occurred, with parallel regenerate columns projecting from each osteotomy surface towards the centre of the regenerate organ. The columns were separated by radiolucent areas with a transverse radiolucent interface at the centre.

The radiolucent band corresponded histologically to parallel bundles of collagen staining fibres, with short strands of spindle cells, all orientated in the direction of distraction. Large peripheral vascular channels were identified, with a similar longitudinal orientation interposed between collagen bundles. Near the vessels, the fibrous matrix condensed around the cells, which stained strongly for calcium. Bone formed parallel to the vascular channels with an alternating pattern of microcolumns extending from the fibrous interzone to each corticotomy surface in the longitudinal section.

The authors observed that both fixators induced a similar degree of osteogenesis when distracted at a similar rate. Stable distraction with either fixator produced intramembranous ossification and the histology of the new bone reflected the different local strains that were predicted for each fixator.

Yasui *et al* (Yasui et al., 1997) investigated the mechanism of bone formation in a Sprague-Dawley rat model using a Hoffman mini-lengthening system. Distraction at 0.25 mm every 12-hours was started 7-days after femoral osteotomy and was

stopped after 21-days distraction. Radiographs were taken each week and animals were killed at intervals.

In-situ hybridisation was performed on de-calcified sections using a digoxigenin labelled cRNA probe for mouse $\alpha 1$, type I and II collagen. Histological examination demonstrated that typical endochondral bone formation was prominent in the early stages of distraction. Intra-membranous bone formation was predominant in the later stages and a third method, which was termed transchondroid bone formation, also occurred. In-situ hybridisation demonstrated that the chondroid cells temporarily expressed type II collagen mRNA but did not show classical morphological characteristics of chondrocytes. It was assumed that these were young chondrocytes undergoing further differentiation into bone forming cells.

Sato *et al* (Sato et al., 1998) used an identical model to investigate the histological and molecular events associated with distraction osteogenesis and demonstrated that the osteotomy site was initially surrounded by hyaline cartilage external callus. During distraction, chondrocytes were stretched along the axis of tension and differentiated into fibroblast like cells. These cells expressed osteopontin, osteocalcin and alkaline phosphatase mRNA's. During the late distraction phase, the cartilaginous callus initially underwent endochondral ossification to form bony callus with later new bone formed by intramembranous ossification. Levels of osteopontin, osteonectin, matrix Gla protein and osteocalcin mRNA expression increased during distraction, suggesting that mechanical environment stress modulated cell shape and phenotype and stimulated the expression of mRNA for bone matrix proteins.

Rowe *et al* (Rowe et al., 1999) investigated the mechanism of angiogenesis in a rabbit mandible model of distraction osteogenesis. The right hemi-mandible was osteotomised and distracted using a customised device at a rate of 0.25 mm every 12 hours for 6-days. Animals were sacrificed at intervals and regenerate histology and intensity of angiogenesis were assessed. There was an intense early vascular response, with abundant new vessel formation during the first 2-days of distraction. This decreased significantly in later stages of distraction and during consolidation.

1.6 Bone Transport

Bone transport describes a technique that uses distraction osteogenesis to reconstruct segmental defects, usually in long bones. Transport is mediated by monolateral or circular external fixators, restoring limb length without requiring massive bone grafting. The transporting segment gradually crosses the defect and regenerate bone is formed according to the principles of Ilizarov. Docking is eventually achieved and the integrity of the bone is restored by a combination of direct bone healing at the docking site and regenerate bone formation in the defect.

1.6.1 Bone Transport for Traumatic Defects

Paley *et al* (Paley et al., 1989) reported 25 patients with tibial non-union associated with a mean bone loss of 6.2 cm. Thirteen of this group had chronic osteomyelitis, 19 had limb length discrepancies, 12 had a bony defect and 13 had deformity.

Management involved internal bone transport with limb length correction by distraction of a percutaneous corticotomy or compression and subsequent distraction of a pseudarthrosis. Infection was treated by radical resection of necrotic bone followed by internal lengthening to reconstruct the defects. Union was achieved in all cases at the mean time of 13.6-months. The results were graded as excellent in 18 cases but there was persistent infection in 3, deformity in 4 and limb shortening in one.

Cattaneo *et al* (Cattaneo et al., 1992) reported internal bone transport in 28 patients with infected non-union or inter-calary tibial bone loss. Six of this group had infected non-union, with hemi-circumferential bone loss; the remainder had an average 4 cm segmental bone loss. The non-unions were treated by en-block resection and internal transport using olive drag wires. Equal limb length and good to excellent function was achieved in 75% of cases.

Green *et al* (Green et al., 1992) reported 17 patients with inter-calary defects managed with an Ilizarov fixator. The mean length of regenerate was 5.14 cm (1.5-14.5) in tibial cases. The mean external fixator time for all patients was 9.6-months (5.5-17.7). The mean time for transport of the moving segments was 4.8-months

(2.3-6.0). The mean distraction index, calculated from the presented data was 0.93 months/cm and the mean external fixator index was 1.87 months/cm.

Bone healing eventually occurred in 94% but complications were common, with an average of 3.5 complications per patient. These included, pin site infection (112%), cellulitis requiring antibiotic treatment (41%) and bone grafting (35%).

Naggar *et al* (Naggar et al., 1993) reported 11 patients with a mean bone defect of 6.7 cm following trauma. The average duration of treatment for this group was 12.9-months, 50% had residual deformity exceeding 5° and 40% had a leg length discrepancy averaging 1.5 cm. Using the figures presented, the mean distraction index was 0.88 months/cm and the external fixator index was 1.93 months/cm.

Prokuski *et al* (Prokuski and Marsh, 1994) reviewed the indications, techniques and results of segmental transport in acute trauma. Transport was considered appropriate for bone loss between 3 to 12 cm and early aggressive infection that required intercalary resection.

Song *et al* (Song et al., 1998) reported the management of 27 tibial defects treated by internal transport using an Ilizarov fixator, followed for an average of 2.5-years.

Satisfactory union occurred in all cases after an average time of 8-months in the fixator with a radiographic consolidation index of 1.3 months/cm. Complications were common and included tibial shortening (37%) ankle stiffness (33%) and malunion (19%)

Paley *et al* (Paley and Maar, 2000) reported 19 patients with tibial defects treated by bone transport using an Ilizarov external fixator with a mean external fixator index of 1.6 months/cm. Debridement of bone ends and bone grafting at the end of transport was required in 53%. Union was achieved in all cases and was complicated with re-fracture at the docking site (5%), limb length discrepancy greater than 2.5cm (5%) and angular deformity greater than 5° (5%).

Rogers *et al* (Rogers et al., 2007) used a stacked Taylor Spatial Frame (TSF) to achieve bone transport in 14 patients, 10 of which had bone loss secondary to

excision of an infected non-union. The mean external fixator time was 12.3-months with an external fixator index of 3 months/cm to reconstruct a mean defect of 49 mm. Unplanned procedures were required in 15 and 8 required a docking procedure. Radiological and functional outcome were evaluated by the Association for the Study and Application of the Methods of Ilizarov (ASAMI) and SF 36 protocols and were rated as excellent in 12 patients (86%).

1.6.2 Bone Transport Following Benign Tumour Excision

Lee *et al* (Lee et al., 2006) reported their experience of treating 16 children with osteofibrous dysplasia. Resection and segmental transport were used in 5 cases, 2 of which had histological evidence of adamantinoma. One patient had a resolving equinus contracture; the remainder were without symptoms or recurrence.

Hahn *et al* (Hahn et al., 2007) outlined the treatment 14 patients with osteofibrous dysplasia at mean age of 13.9-years (2-65). One patient aged 2-years was treated with a segmental excision and bone transport using an Ilizarov fixator. This resulted in recurrence 18-months later, which was treated with curettage and grafting and with no further evidence of disease at 11-years.

1.6.3 Bone Transport Following Malignant Tumour Excision

Segmental transport has previously been used after excision of malignant bone tumours; reports however, are usually single cases or small series.

Lenoble *et al* (Lenoble et al., 1995) reported simultaneous bone and soft tissue transport to reconstruct tissue loss in the lower leg. The series considered 12 patients, 2 of which had en bloc resection of bone, muscle and skin for malignant bone tumour. There are no details of the type of tumour or results in these individual cases. This paper does not allow any further sensible comment on the use of distraction osteogenesis in the management of bone tumours.

Carter *et al* (Carter et al., 1999) reported the treatment of a low grade leiomyosarcoma in the mandible of a 7-year old girl. This was treated by incision biopsy and subsequent neck dissection with tumour excision and mandibular reconstruction using a dynamic reconstruction plate followed by a multi-

dimensional distraction device. Bi-focal distraction osteogenesis was performed to bridge a 4 cm gap and this was subsequently augmented with an iliac crest graft. This patient did not receive chemotherapy as part of the tumour management.

Iacobellis *et al* (Iacobellis and Olmeda, 2004) described the treatment of a mesenchymal chondrosarcoma and 2 cases of squamous skin carcinoma with tibial infiltration. The mesenchymal chondrosarcoma occurred in a male aged 14-years who was treated with bone transport and tibio-talar arthrodesis. This patient underwent cyclical chemotherapy during the course of the distraction and had slow corticalisation of the regenerate with no evidence of recurrence at 10-years. The cases of squamous skin carcinoma were treated with bi-focal transport without chemotherapy, followed up for 2 and 3-years with no evidence of recurrence.

1.6.4 Bone Transport in the Management of Ewing's and Osteogenic Sarcoma with simultaneous Cytotoxic Chemotherapy

Lammens *et al* (Lammens and Fabry, 1992) included one patient with osteogenic sarcoma of the ulna in a report of 6 cases. Resection resulted in a 10 cm defect, which was reconstructed with proximal corticotomy and bone transport. The external fixator index was 24 days/cm, resulting in good function with no evidence of relapse. There was however a fibrous non-union, which was pain free and did not cause any functional impairment.

Naggar *et al* (Naggar et al., 1993) included one patient with an osteogenic sarcoma treated with an Ilizarov fixator in a series of 11 patients. The defect was 18 cm with transport occurring over 5-months and consolidation over 11-months. There was no documentation of complications associated with this case.

Canadell *et al* (Canadell et al., 1994) used physeal distraction to expand the tumour free margin and preserve the epiphysis in 7 patients with Ewing's and 13 with osteogenic sarcoma. The paper includes details of the operative technique, which initially involved physeal distraction over 15 days to increase the tumour free margin by 2 cm. The tumour was resected en bloc and provided margins were clear, the defect was reconstructed with autograft from the contra-lateral tibia or allograft. There were no local recurrences in the epiphyseal region and one

diaphyseal recurrence at a mean follow-up of 54-months but 3 patients had died from pulmonary metastases.

Said *et al* (Said and el Sherif, 1995) reported two cases in which limb reconstruction was accomplished by resection- shortening- distraction. Case 1 involved a 14-year old male with Enneking Stage IIB osteogenic sarcoma. Pre-operative chemotherapy was given for five weeks (Glasser and Lane, 1991) prior to en bloc excision including the knee, resulting in a 17 cm defect. This was closed using a Hoffman external fixator with eight cycles of post-operative chemotherapy. The external fixator was removed after bone union and initial management was with a 15 cm shoe raise. A corticotomy was performed after completion of chemotherapy and 9 cm lengthening was achieved with a Wagner external fixator, complicated by a popliteal nerve palsy and severe equinus deformity. Case 2 involved a 41-year old female with a malignant fibrous histiocytoma of the lower femur, which was resected *en bloc* with the knee leaving an 11 cm defect. The bone ends were brought together using an Ilizarov fixator and distraction osteogenesis was performed to a total of 9 cm.

Tsuchiya *et al* (Tsuchiya et al., 1997) reported segmental transport, shortening distraction and distraction over an intramedullary nail to reconstruct defects produced after excision of malignant bone tumours. Segmental transport was used in 10 cases (5 osteogenic sarcomas, 5 giant cell tumours) using a monofocal osteotomy with iliac bone grafting at the docking site. The tumours were proximal tibial (8), midshaft tibial (1) and distal femoral (1). The average defect was 8.8 cm and 3 patients had chemotherapy during distraction.

The mean external fixation index was 39.4 days/cm, with a total fixator time of 251-days for the tibial transport group and 440-days for the femoral transport group. Significant complications were reported and included skin invagination (2), pes equinus (2), premature consolidation (1), fracture at the docking site (1), subluxation of the head of the fibula (1).

Forty-three percent of patients who received chemotherapy during distraction osteogenesis were dead at a mean time of 29-months. This is significantly worse than would be expected with the contemporary management of osteogenic

sarcoma and suggests that the reconstruction technique modified the outcome. This may be due to compromising resection margins, activating the tumour with distraction osteogenesis or may be un-representative, given the small sample size.

Canadell *et al* (Canadell et al., 1998) described a series of 61 cases of bone tumors managed with an external fixator. These were sub-divided into pathological fractures managed with external fixation and patients treated by external fixation at an interval after the primary oncological surgery to deal with bone lengthening, limb shortening, pseudarthrosis, infection and correction of angular deformities. A separate group is described in which an external fixator was used to maintain position of an autograft or to reconstruct a bone defect with physeal distraction or bone transport.

Physeal distraction was carried out in 22 patients, 14 with osteogenic sarcoma and 18 with Ewing's sarcoma. In each case, the tumour was metaphyseal and proximal to the physeal cartilage, which was unaffected. Distraction was performed using external fixator at the rate of 1 to 2 mm/day until a gap of approximately 2 cm was created. The fixator was left in situ for 10-20 days and the result was reported to be good in all patients. It is not possible to assess the treatment within these 3 groups from the data presented, as the patient demographics relate to the whole group.

The details of bone transport in a 5-year-old girl with an osteogenic sarcoma of the distal femoral metaphysis is described separately. Bone transport was effected using an Limb Reconstruction System (LRS) fixator, which was worn for 235-days. There is no information describing the chemotherapy regimen and insufficient clinical detail to allow any more sophisticated discussion of the role of bone transport in the situation. The recommendation was that bone transport should be conducted in very young patients with small diaphyseal and distal metaphyseal tumours of the femur.

Ozaki *et al* (Ozaki et al., 1998) described the treatment of 5 patients with bone sarcomas (3 osteogenic sarcomas, one Ewing's and one chondrosarcoma) managed by bi-focal bone transport. The mean skeletal defect after tumour resection was 17 cm (10-25). The mean duration of external fixation was 32-

months (579-1340 days) and the mean treatment index was 95 days/cm. (53.5-191). The average follow up period was 48-months (40-66). Satisfactory bone formation was only achieved in one case with significant functional impairment in all others. There were frequent complications, which included re-absorption of regenerate, skin necrosis, non-union, fixation failure, pin track infection and persistent equinus. In view of the poor experience the authors did not advise this as a method of reconstruction for bone sarcomas.

Kapukaya *et al* (Kapukaya et al., 2000) reported 9 patients with femoral tumours, including one stage IIB Ewing's and 3 stage IIB osteogenic sarcomas. All 4 received pre and post-operative chemotherapy although the regimen is not specified.

The mean age of the osteogenic sarcoma group was 9.6-years (8-13) and the mean skeletal defect was 10.7 cm (10-12 cm). The mean distraction rate was 14.0 days/cm (12-15.7) and the mean external fixation index was 33.4 days/cm (32.5-34.5). The mean period of follow up was 33-months (20-30) and at the time of follow up one patient had died of their disease.

The Ewing's sarcoma was in a 7-year-old female with a skeletal defect of 13 cm with a distraction index of 12.7 days/cm and an external fixator index of 32.8 days/cm. There was no evidence of recurrent disease or significant complication at 20-months.

There were 6 patients who did not receive chemotherapy and the authors noted that there was no difference in external fixator index between groups. They recognised that the numbers in this study were too small to allow a definitive opinion on this point.

Millett *et al* (Millett et al., 2000) reported 2 cases aged 14 and 26-years, with a mean follow-up of 24-months. Reconstruction was with bone transport using a unilateral external fixator to bridge a bone defect of 15 and 18 cm.

The external fixator index was 30 and 38.5 months/cm and the distraction index was 11 and 15.7 months/cm. Both patients had a good function with no evidence of relapse and complications included premature consolidation and deep infection.

Tsuchiya *et al* (Tsuchiya et al., 2002) reported the results of 11 patients with peri-articular osteogenic sarcomas treated with intra-epiphyseal excision of the tumour and reconstruction by distraction osteogenesis. Eight of this group, with high-grade tumours received pre- and post-operative chemotherapy (5 cycles of intra-arterial cis-platinum, caffeine and adriamycin at intervals of 3-weeks).

Evidence of good response consisted of sclerotic changes or good margination of the tumour on plain radiographs, marked shrinkage of any extension of the tumour into soft tissues on Magnetic Resonance Imaging (MRI) and disappearance of tumour vessels on angiography. Patients with at least 1cm of preserved epiphysis were considered for intra-epiphyseal excision with reconstruction using conventional bone transport, shortening distraction or bone transport after insertion of a diaphyseal bone cylinder. Distraction was started between 1 to 2-weeks after surgery at a rate of 1mm/day.

Chemotherapy was continued during the post-operative period for patients with high-grade tumours using 3 courses of intravenous cis-platinum, caffeine, adriamycin, vincristine, high dose methotrexate and citrovorum factor. Two patients had died at the time of follow-up, one from the sarcoma and one from hepatitis. The remainder had a continuous disease free interval average of 59-months. This group had a mean lengthening of 9 cm with a mean distraction index of 14 days/cm and an external fixation index of 35 days/cm. Normal limb function was restored in 8 of the 9 surviving patients, 3 had initial shortening, 2 of whom required further lengthening and one required a shoe-lift.

Laitinen *et al* (Laitinen et al., 2005) reported their experience of 15 patients with primary malignant tumours of the distal tibia, 14 of which were treated with limb salvage surgery. Reconstruction was achieved by tibio-talar arthrodesis in combination with a variety of techniques, with 3 cases managed by bone transport. Patients received adjuvant or neo-adjuvant chemotherapy according to the Co-operative Osteosarcoma Study (COSS) for osteogenic sarcoma and European-

Ewing study for Ewing's Sarcoma. Bone transport was not started until chemotherapy had been completed and a cement spacer was inserted into the resected area as an interim measure. The mean defect was 12.8 cm (12.5-13) and reconstruction was combined with an arthrodesis.

Two female patients (14 and 16-years) had Enneking stage 11B osteogenic sarcoma with a Salzer-Kuntschik (Salzer-Kuntschik et al., 1983) grade 1 response to chemotherapy. At follow-up of 1.3 and 5.1-years, there had been wound healing problems that had been managed conservatively. There was no evidence of recurrent disease in either patient.

A 37-year old male with Ewing's sarcoma Grade 11B also had a grade 1 response to chemotherapy. There was no evidence of disease 9.1-years after initial presentation. Treatment was complicated by non-union of the bone transport and infection. The treatment was initially with a fibular transfer and subsequently an amputation.

1.6.5 Distraction Osteogenesis to Manage Limb Length Discrepancy in Patients Previously Treated with Cytotoxic Chemotherapy

Cara *et al* (Cara et al., 1993) reviewed their experience of surgical lengthening in 8 patients who had previously undergone treatment for Ewing's or osteogenic sarcoma. There is very little detail about the primary treatment and no information describing the prior use of chemotherapy. Lengthening was conducted with a mono-lateral (Wagner or Monotube LC) fixator at an unspecified time following primary treatment. The average discrepancy was 12.6 cm and equalisation was achieved in five patients. The authors state an interval of three years from completion of multi-disciplinary therapy should be allowed before limb lengthening is undertaken but there is no data in the text to validate this statement.

Gonzalez-Herranz *et al* (Gonzalez-Herranz et al., 1995) presented 15 cases of lower limb length discrepancy greater than 5 cm in patients who had undergone treatment for Ewing's or osteogenic sarcoma. The type and duration of chemotherapy and the interval between tumour treatment and limb lengthening is not specified.

Ten patients were managed by surgical lengthening, 3 with a second lengthening using an Orthofix fixator (11) and Ilizarov fixator (2). The average lengthening was 8.1 cm with a healing index of 41 days/cm (31-56). The authors make the point that patients that have previously undergone field irradiation are unsuitable for surgical lengthening due to “irreversible lesions in the bone matrix”.

Catagni *et al* (Catagni et al., 2003) used the Ilizarov method to correct limb length discrepancy in a 26-year old patient who underwent a hemi-pelvectomy for a Ewing’s sarcoma 15-years previously. Neo-adjuvant chemotherapy (Vincristine, Cyclophosphamide, Doxorubicin and Actinomycin D) had been completed 12-years previously and there was no evidence of local recurrence. There was 6 cm residual shortening with tibio-femoral axis mal-alignment.

Reconstruction was achieved with a 3 level hybrid Ilizarov fixator, allowing simultaneous femoral and tibial/fibular lengthening to a total of 6 cm.

There was evidence of clinical and radiological consolidation 6-months after fixator application and a good functional outcome with unaided walking.

Ilizarov *et al* (Ilizarov et al., 2007) described a patient who was initially treated for an osteosarcoma of the proximal humerus. Tumour resection was followed by shoulder hemi-arthroplasty, revised 10-months later to an arthrodesis with a vascularised fibular graft. There was no detail of the chemotherapy associated with the initial tumour treatment. Nine-years after treatment a 9 cm discrepancy was corrected using a unilateral device with an external fixator index of 1.33 cm/month. This was complicated with an oblique fracture in the central part of the regenerate, which was treated in a brace and subsequently with internal fixation.

Oh *et al* (Oh et al., 2008) reported the use of sub-muscular plating after distraction osteogenesis to reconstruct segmental defects in children. Two required bone transport for a tibial and femoral defect. The external fixator index for the femoral transport was 14.2 days/cm, with a healing index of 46.5 days/cm. The healing index for tibial transport was 39.6 days/cm, with a healing index of 66.4 days/cm. There are no details of tumour type or the use of cytotoxic drugs and it is therefore not possible to make any comments relating to the behaviour of regenerate in these patients.

Romanos *et al* (Romanos et al., 2008) reported a 13-year old female with a massive knee endoprosthesis after excision of a proximal tibial osteogenic sarcoma. There was a 9.5 cm limb length inequality, which was managed with attempted tibial lengthening with an Ilizarov fixator over the stem of the prosthesis. A 5cm lengthening was achieved with good quality regenerate. The procedure was however complicated by a severe infection, which did not respond to antibiotics and led to removal of the fixator, removal of the prosthesis with a methylmethacrylate spacer and an above knee cast.

Eralp *et al* (Eralp et al., 2009) reported 8 patients with infection or deformity following surgery, 6 patients with shortening secondary to tumour surgery and 4 patients with deformity and shortening secondary to multiple exostoses and Ollier's disease.

In the first group, there were 3 patients with Ewing's and one with osteogenic sarcoma. All were treated for the complications of primary salvage surgery and at the time of external fixator surgery, were not currently or had not recently been treated with cytotoxic drugs.

In the second group, there was one patient with a Ewing's sarcoma and one with osteogenic sarcoma initially treated with a fibular graft resulting in non union and shortening. These patients were successfully treated with an external fixator 12 and 144-months after primary treatment. This paper is therefore a technical report rather than a description of the management of the tumour, which it can be assumed, was cured by the primary treatment.

Courvoisier *et al* (Courvoisier et al., 2009) described lengthening of vascularised free fibular grafts in 3 patients, 2 who had previously undergone resection for chondroblastic osteogenic sarcoma and Ewing's sarcoma of the tibia, The histology of the patient with chondroblastic osteogenic sarcoma was subsequently reviewed and was judged to have been a chondromyxoid fibroma

This series is therefore relevant only in that one patient who had Ewing's sarcoma underwent distraction osteogenesis in a vascularised fibular graft at a point that they had effectively recovered for their primary tumour and initial treatment.

The authors highlighted major concerns associated with this type of approach and in particular, considered that whilst bone lengthening over a prosthesis stem is possible and may produce a good quality regenerate, it should be avoided due to the high risk of secondary bone infection.

1.6.6 Overview of the Role of Distraction Osteogenesis in the Management of Malignant Bone Tumours

Distraction osteogenesis in general and bone transport in particular has been reported in a number of previous publications discussing the management of malignant bone tumours. These contain small numbers and often include cases of malignant bone disease in a more broad description of technique. Other series describe strategies that use an external fixator in the tumour patient as an adjunct to alternative methods of reconstruction and in late reconstruction following the complications of primary treatment.

It is clear from the previous literature that both traumatic and tumour cases are associated with significant complications with infection and mal-union being common. It is difficult to make direct comparisons due to the disparate nature and small numbers in each of the series considered. The series are non-standardised in terms of primary diagnosis, external fixator type and definition of each phase of treatment and it is not possible to draw robust conclusions. There is no previously published data that specifically considers the effect of cyclical cytotoxic chemotherapy on bone formation in human subjects.

1.7 The Aim of This Thesis

Thirty years ago, the outcome for patients with osteogenic sarcoma was bleak. The likelihood of survival beyond five years was of the order of 20 – 25%, with death predominantly being due to metastatic disease. The introduction of cytotoxic chemotherapy led to a rapid and significant improvement in survival. An appreciation that survival was not influenced by the choice of treatment for local disease led to further important developments and in 1986, Simon *et al* (Simon et al., 1986) demonstrated equivalent survival for patients undergoing amputation or a limb sparing procedure. This stimulated advances in the surgical management of local disease with modification of conventional techniques. Biological or

endoprosthetic reconstruction techniques are prone to infection, lack of biological affinity or lack of durability.

Distraction osteogenesis is widely used to manage intercalary defects following trauma and infection and could potentially provide a superior method of reconstruction. The concomitant use of chemotherapy in this situation however, adds an extra dimension to the complexity and although there are several reports, this technique is not in widespread use.

The effect of cytotoxic drugs on regenerate bone is unknown and the efficacy of distraction osteogenesis in the management of malignant tumours is therefore uncertain. This thesis will attempt to address this specific deficiency in knowledge from two directions.

The first pillar of this research is the development of a robust experimental model, which can be used to investigate the effects of cytotoxic chemotherapy on distraction osteogenesis.

The second pillar uses this model to investigate the effect of cytotoxic drugs on the structural and material properties of the regenerate bone.

1.7.1 General Hypothesis

The general hypothesis is that it is possible to produce structurally competent bone by distraction osteogenesis in the presence of cyclical cytotoxic therapy.

1.7.2 Deliverable Objectives

Pillar 1 Development of an Animal Model

An investigation was performed to develop a NZW rabbit model that used a combination of cis-platinum and adriamycin to consistently produce a clinically relevant cytotoxic effect without inducing renal or hepatic failure.

Previous studies were concerned primarily with the pharmacokinetics and toxic effects of these drugs, using high doses with short periods of assessment. There was an absence of data indicating doses relevant to this investigation, the

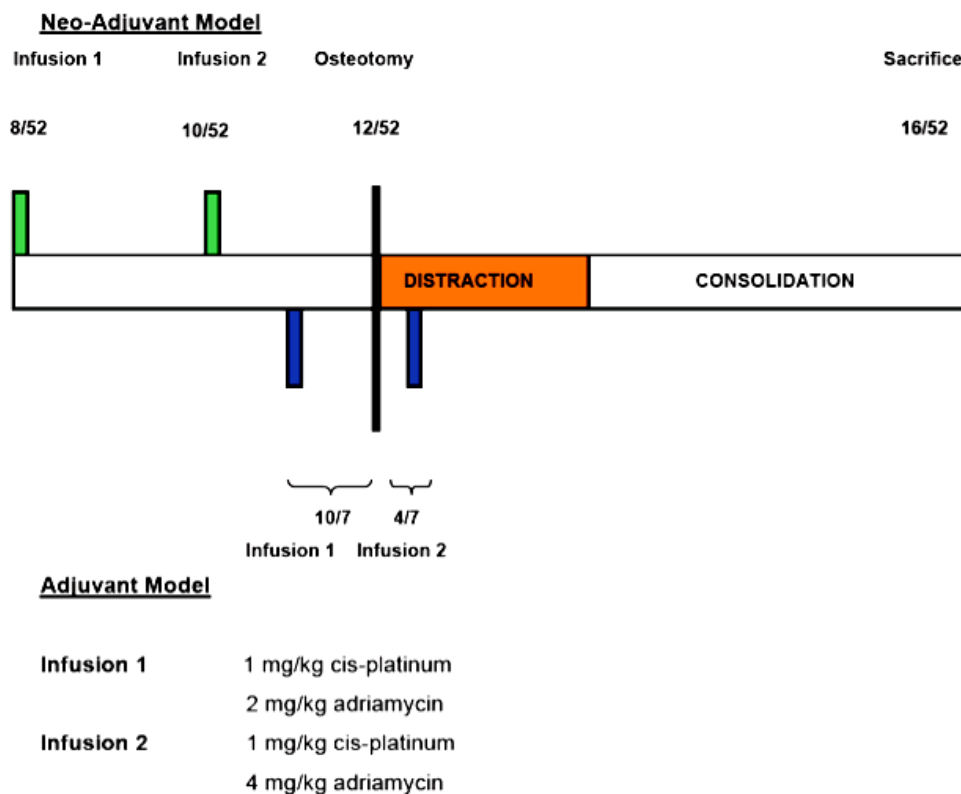
optimum route of administration and the combined effect of cis-platinum and adriamycin on renal, cardiac and general metabolic function in this species.

An appropriate chemotherapy schedule was identified that met the conflicting requirements of producing a measurable cytotoxic effect, without adversely affecting metabolic function. A standard method of cytotoxic delivery was developed using an infusion pump to deliver drugs via the femoral vein.

Pillar 2 *Investigation of the Effect cis-Platinum and Adriamycin on the Structural and Material Properties of Bone Formed by Distraction Osteogenesis.*

Two cycles of either cis-platinum and adriamycin or normal saline were given to juvenile NZW rabbits that subsequently underwent limb lengthening using a mono-lateral external fixator aged 12-weeks.

The experiment model used either 2-cycles of drugs given 4 and 2-weeks before osteotomy and distraction (Neo-adjuvant Group) or one cycle 10-days before and 4-days after osteotomy and distraction (Adjuvant Group).



The lengthened tibia was evaluated using plain radiography, DXA and compression testing to determine differences in the structural and material properties of the regenerate that could be reasonably attributed to the presence of cytotoxic drugs. The relationship between the timing of administration of the drugs and effect on the regenerate was also evaluated.

Section 2 Development and Validation of the Animal Model

The first pillar of the experiment involved the development of a NZW rabbit model in which a clinically relevant cytotoxic dose was infused whilst preserving normal renal and hepatic function. The compounding effect of deranged metabolic function would make interpretation of subsequent data impossible and preservation assumed primacy over the level of cytotoxic effect. The working compromise was to deliver maximum doses of cis-platinum and adriamycin compatible with normal renal and hepatic function, with a < 5% rate of infection. The dose at which this was achieved was the maximum that could be used in the next stage of the experiment. Reduction in percentage weight gain was taken as evidence of an adequate cytotoxic effect, similar to that which would be seen in a human patient and previously described in the NZW rabbit. (Wanless et al., 1987, Young et al., 1975)

This chapter considers published studies that report the metabolic effects of cis-platinum and adriamycin on this species and the effect of cytotoxic drugs on distraction osteogenesis. This resulted in the development of an experimental model, which satisfied the following criteria;

1. Reproducible anaesthesia
2. Reproducible infusion of cis-platinum and adriamycin
3. Appropriate cytotoxic effect
4. Normal renal function
5. Normal hepatic function

2.1 Studies using Adriamycin

Young et al (Young et al., 1975) infused 8-week old NZW rabbits with adriamycin (0.7 mg/kg) 3 times per week for up to 4-months with a control group receiving equivalent volumes of saline. There was a significant mortality in the treated group, with 57% (8/14) dying of drug toxicity before the conclusion of the experiment. Post mortem analysis demonstrated severe cardiomyopathy and glomerulonephrosis. The remaining treated animals had evidence of myocardial damage and mild to moderate glomerulonephrosis. There was a significant increase in BUN, reduction in alkaline phosphatase levels and the rate of weight gain decreased in the treated group, with terminal weights 61% of control animals.

There were structural alterations in the skeleton of the treated group, with reduction of cortical thickness to 82% of controls. The distal femoral physis was radiologically indistinct, corresponding to histological differences including loss of the columnar arrangement in the zone of proliferation. The zone of provisional calcification was reduced in all treated bones and there was a paucity of vascularised connective tissue, osteoblasts and osteoclasts. There was a reduction in numbers of bony trabeculae in the primary and secondary spongiosa and trabeculae were frequently disorientated from the longitudinal axis of the bone. Cells surrounding the trabeculae were spindle to ovoid shaped and were considered to be populations of fibroblasts and resting stem cells. There was mild to moderate marrow hypoplasia with replacement fibrosis, which was particularly prominent in the metaphysis of animals treated for longer periods. The cortical bone from treated rabbits appeared histologically immature compared to age matched controls

Jaenke *et al* (Jaenke, 1976) infused mixed sex NZW rabbits with adriamycin (2.25 mg/kg/week in 3 divided doses) which were assessed after 23, 35, 43 and 75-days of continuous treatment. Adriamycin toxicity was assessed by total leukocyte count, haematocrit, creatine phosphokinase (CPK), lactate dehydrogenase (LDH) and weight gain. Myocardial lesions were described using qualitative and quantitative methods of microscopic assessment. Group B closely mimicked the experimental requirements of this study and received a total dose of 6.75 mg/kg during 23-days of treatment. In this group, there was an early rise in CPK and LDH levels, which continued throughout treatment, gradually normalising approximately 10-weeks after cessation of adriamycin. There was no histological evidence of immediate or late (53-days) cardiac damage.

Bertazzoli *et al* (Bertazzoli et al., 1976) investigated the cumulative effect on male NZW rabbits of an infusion of adriamycin (0.8mg/kg), 3 times per week for 7-weeks compared to normal saline controls. There was histological evidence of myocardial degeneration in the treated group with decreased activity of succinate dehydrogenase CoQ₁₀ indicating significant, cumulative cardiotoxicity.

Arnolda *et al* (Arnolda et al., 1985) investigated the effect of chronic intravenous adriamycin (1mg/kg) administration twice weekly for 8-weeks in mixed strains

including NZW rabbits. This dose schedule was designed to produce minimal marrow toxicity and is a modification of the technique described by Jaenke *et al.* (Jaenke *et al.*, 1980) All animals developed cardiac failure within 8-weeks with *post mortem* evidence of cardiac dilation, pleural and pericardial effusions, ascites and hepatic congestion.

Wanless *et al* (Wanless *et al.*, 1987) infused adult NZW rabbits with adriamycin (1mg/kg) via an ear vein, twice per week for 6, 8 or 10-weeks with control groups receiving no treatment. Assessment included simple clinical, haematological and biochemical investigations in addition to invasive cardiovascular monitoring. The treated group continued to increase in weight but at a slower rate than the control group. There was a significant reduction in haemoglobin and a non-significant decrease in white cell count, both these parameters returned to control levels in animals treated for 8-weeks with 2-weeks to recover. There was a variable, dose dependant effect on heart rate (HR), cardiac output (CO) and total peripheral resistance (TPR) with reduction of CO in all groups and a reduction of TPR in the 8 and 10-week groups.

Jones *et al* (Jones *et al.*, 1990) infused NZW rabbits of unspecified age with adriamycin (1mg/kg) twice per week for 8-weeks with 2-weeks recovery. Outcome measures included enzymic analysis of cardiac myosites and measurement of contraction amplitude and velocity. After 8-weeks of adriamycin therapy, all animals had a cardiomyopathy similar to that occurring in human patients with adriamycin cardiotoxicity. There were *post mortem* signs of severe congestive cardiac failure with biventricular dilatation, pleural and pericardial effusions, ascites and hepatic congestion. There was histological evidence of widespread atrophy and lysis of the cardiac myocytes associated with interstitial fibrosis.

Calderone *et al* (Calderone *et al.*, 1991) identified a potential cellular mechanism of cumulative cardiotoxicity using juvenile NZW rabbits infused with adriamycin (0.75 mg/kg) 3 times per week for 11-weeks. This study demonstrated that adriamycin administration caused early dysfunction of the myocardial β -adrenergic system due to a decrease in adenyl cyclase activity.

Cusack *et al* (Cusack *et al.*, 1992) compared the effect of adriamycin on normally nourished and malnourished adult NZW rabbits weighing between 2.5 and 3.5 kg.

The normally nourished group is comparable to the experimental group in this thesis. Animals received 5 mg/kg adriamycin via an ear vein as a bolus over 2-minutes. Plasma concentrations of adriamycin (doxorubicin) and the principle metabolite, doxorubicinol were obtained at intervals up to 52-hours after infusion. Malnourishment had a significant effect on the pharmacokinetics of both compounds but the duration of the experiment was short and did not include histological or haemodynamic assessment of renal or cardiac function.

Bocherens-Gradient *et al* (Bocherens-Gradient *et al.*, 1992) used male rabbits to investigate the effect of intravenous adriamycin (1mg/kg) twice per week for 9-weeks. In this study, 11/15 animals did not survive to complete the experiment, 5 due to the direct effects of adriamycin. All 4 survivors demonstrated signs of advanced cardiac failure but the severity of symptoms varied, indicating differences in the individual sensitivity of the animals to adriamycin.

Cusack *et al* (Cusack *et al.*, 1993) compared the pharmacokinetics of adriamycin (doxorubicin) and doxorubicinol in young male NZW rabbits infused with 5 mg/kg adriamycin by 2-minute bolus or 24-hour continuous infusion. Blood was sampled at intervals during infusion or following bolus administration. There was a significant increase in peak left ventricular tissue concentration following bolus administration but follow-up time was short and the study did not include histological or haemodynamic assessment of cardiac failure.

Dodd *et al* (Dodd *et al.*, 1993) infused adult NZW rabbits with adriamycin (1mg/kg) over 30-minutes via an ear vein twice weekly for a total of 12 to 18 doses and compared this to a saline infused control group. There was a significant increase in ventricular weight in the treated group and an increase in heart/body weight ratio. Microscopic changes in the ventricles included sarcotubular swelling without mitochondrial or myofibrillar loss and changes in papillary muscles consistent with adriamycin cardiomyopathy. The study identified a further potential mechanism of adriamycin cardiotoxicity that involved a decrease in calcium release channels in the sarcoplasmic reticulum.

2.2 Studies using cis-Platinum

Schiffer *et al* (Schiffer et al., 1996) studied central nervous system toxicity in a NZW rabbit model by injecting a single 2 mg/kg dose of cis-platinum into the left carotid artery. There were no demonstrable clinical effects on the animals and no neurological abnormalities. There was no demonstrable effect on brain histopathology following the administration of cis-platinum but neither renal function nor histology was evaluated.

Frank *et al* (Frank et al., 1989) used gadolinium-DTPA enhanced dynamic MRI to monitor the nephrotoxic effects of cis-platinum in NZW rabbits. Animals were divided into 2 groups and given either 8 mg/kg (Group 1) over 1-hour or 3 mg/kg (Group 2) each day for 5 consecutive days. In group 1 there was MRI evidence of nephrotoxicity within 9-hours of administration and an increase in Blood Urea Nitrogen (BUN) and creatinine within 48-hours. The acute toxic effects of cis-platinum led to severe anorexia and weakness and necessitated killing the animals by day-7. In group 2, the animals received a higher cumulative dose and demonstrated a marked deterioration in weight, but a less severe rise in BUN and creatinine.

Histology of the kidneys from both groups demonstrated mild to moderate foci of necrosis of the PCT with proteinaceous material in the PCT extending to the loop of Henlé. Focal areas of cystic dilatation of the proximal tubules and areas of lymphocytic infiltration were also seen in the rabbits in group 2 and possibly represented the late effect of cis-platinum nephrotoxicity.

Najjar *et al* (Najjar and Saad, 2001) used adult male NZW rabbits to investigate the effect of a single intravenous 5 mg/kg dose of cis-platinum. Normal rabbits were compared with streptozotocin induced diabetic rabbits 3-days after the induction of a diabetic state. Indices of nephrotoxicity (serum creatinine, BUN and serum albumin) were obtained at 0 and 7-days after cis-platinum administration for both groups. Comparisons between groups 7-days after cis-platinum treatment demonstrated highly significant elevations in serum creatinine and urea and a decrease in serum albumin levels in the non-diabetic group

2.3 Rabbit Models Assessing the Effect of Cytotoxic Drugs on Distraction Osteogenesis

Prevot *et al* (Prevot et al., 1988) used an adult rabbit model to investigate the effect of methotrexate or methotrexate and adriamycin during tibial lengthening. Although consolidation was slightly delayed in the methotrexate group when compared to controls (average 9.5-weeks vs. 9.3-weeks), the difference was not statistically significant. The combination of methotrexate and adriamycin had a more pronounced negative effect on bone regenerate, although significant marrow and cardiac toxicity resulted in early death of numerous animals in the group. This study presented only qualitative histological and radiological analysis and did not specifically address the local or systemic complications that were encountered.

Jarka *et al* (Jarka et al., 1998) investigated the effect of methotrexate in a NZW rabbit model of distraction osteogenesis using a ring fixator. The latent period was one-week, distraction continued at 0.5 mm twice daily for 3-weeks with sacrifice at 28-days or 70-days. Haematological, biochemical and radiological evaluation was performed at intervals until sacrifice with *post mortem* histological examination of the lengthened tibia. Seventeen of 18 rabbits that received 130 mg/kg methotrexate gained weight, thrived and completed distraction. Radiographs were coded and evaluated blindly, with initial bone bridging observed at 4-weeks in all animals except one in the 28-day experimental group. In the 70-day group, consolidation was observed at a mean of 6.4-weeks in the control group and 7.3-weeks in the chemotherapy group and was not statistically significant. There was no difference in haemoglobin concentration or leukocyte count at day 20 or day 70. The calcium and phosphorous concentration in bone regenerate of treated animals was slightly higher at 28-days and slightly lower at 70-days but neither of these findings was statistically significant. There was no quantitative or semi-quantitative difference between the histological findings of the bone in the experimental or control group.

2.4 Large Mammal Models Assessing the Effect of Cytotoxic Drugs on Distraction Osteogenesis

Burchardt *et al* (Burchardt et al., 1983) investigated the effect of adriamycin and methotrexate on the healing of segmental defects in dogs. Adriamycin was given at doses of 30, 40 or 50 mg/m² and compared with control and methotrexate

treated animals 6-months after repair of a segmental defect with a fibular autograft. There was evidence of decreased new bone formation and an increased rate of non-union which was not dependant on the dose of adriamycin. Animals treated with 40 or 50 mg/m² had disproportionate suppression of new bone formation at the periphery of the graft. The combination of new bone suppression and peripheral graft reabsorption resulted in a reduction in torque to failure with the higher doses.

Ehrhart *et al* (Ehrhart et al., 2002) investigated the effect of cis-platinum on bone transport in a canine radius model. Five animals received 4 cycles of cis-platinum (70 mg/m² i.v.) with 5 being given 0.9% saline control. DXA analysis was performed 24, 55 and 90-days after surgery, at which point the dogs were sacrificed. Histomorphometry was performed on non-decalcified sections of regenerate and the BMD and indices of newly formed bone were compared between groups. There was no difference in the BMD between groups but the cis-platinum treated dogs had a decreased mineralised bone volume, decreased percentage of woven bone volume, decreased percentage of osteoblast covered bone, increased porosity and increased percentage of osteoblast covered surfaces compared with the controls. The lamellar bone volume and osteoid volume did not differ significantly between groups. This demonstrated that regenerate bone would form and remodel following the administration of cis-platinum. Histomorphometric analysis suggested an uncoupling of bone formation and reabsorption resulting in increased osteoclast activity or delayed secondary bone formation during remodelling.

Gravel *et al* (Gravel et al., 2003b) investigated the effect of adriamycin in a goat model of tibial lengthening. Animals received an initial dose of 60 mg/m² of adriamycin 6-weeks before surgery with the second dose 3-weeks later. Tibial lengthening was performed after a 5-day lag period with an Orthofix bone lengthener at a rate of 1mm per day for 20-days. The animals were sacrificed at 6, 12 and 24-weeks and the lengthened tibiae underwent radiological, biomechanical and histological analysis. There was no significant difference in radiological histological and mechanical parameters between adriamycin treated and control groups. There was no evidence that chemotherapy affected the healing time or the torsional properties of the bone

2.5 Summary of Available Literature

The published literature describing the use of adriamycin in the NZW rabbit involves either a short interval to sacrifice or omits histological or haemodynamic data. (Arnolda et al., 1985, Bertazzoli et al., 1976, Jaenke, 1976, Jaenke et al., 1980, Young et al., 1975) These studies demonstrate that either cumulative low (1 mg/kg) or bolus high (5 mg/kg) doses predictably induce cardiac failure. Most of these experiments involved a deliberate attempt to produce cardiac failure in a variety of circumstances, whereas preservation of cardiac function was required for the experimental component of this thesis. Jancke *et al* (Jaenke, 1976) demonstrated that intravenous infusion of adriamycin (2.25 mg/kg/week in 3 divided doses) caused a transient rise in CPK and LDH but no histological evidence of myocardial damage at 53-days and provides a potentially safe schedule for adriamycin in isolation.

Similar limitations relate to previous experiments using the NZW rabbit to evaluate cis-platinum and initial experiments involved high doses of cis-platinum. (Frank et al., 1989) Najjar *et al* (Najjar and Saad, 2001) demonstrated that doses of 5 mg/kg predictably produced renal failure but there is no good data to suggest a dose that will preserve renal function in this species.

Previous studies have evaluated the use of single cytotoxic agents on bone healing (Burchardt et al., 1983) and bone transport (Ehrhart et al., 2002) in a canine model and limb lengthening in a goat model. (Gravel et al., 2003b) Methotrexate in isolation (Jarka et al., 1998) and in combination with cis-platinum (Prevot et al., 1988) has been evaluated to investigate the effect on bone formation in the NZW rabbit. There is no previous experimental work that considers the combined effects of cis-platinum and adriamycin on bone formed by distraction osteogenesis.

The initial experimental component of this thesis investigated the metabolic effect of variable doses of cis-platinum and adriamycin on the juvenile NZW Rabbit. The aim was to produce a model that fulfilled the conflicting requirements of a therapeutically relevant anti-neoplastic effect without significant side effects.

2.6 Experimental Considerations

The cytotoxic dose restrictions associated with this experiment have been outlined in previous paragraphs. The working compromise was to deliver the maximum amount of cis-platinum and adriamycin compatible with normal hepatic and renal function, with a < 5% rate of infection. There were no previous studies that used this combination in the NZW rabbit and a study was undertaken to determine the dose that produced a 5% reduction in percentage weight gain. This was considered to be evidence of a relevant cytotoxic effect, which would imitate some of the effects that would be expected in a human patient.

2.6.1 Breeding and Husbandry

All animals were obtained from the Combined Universities Laboratory Animal Services (CULAS), Little Bay, New South Wales (NSW) 2036, Australia. The CULAS NZW Rabbit colony is an outbred Specific Pathogen Free (SPF) colony. The colony consisted of 4 bucks and 22 does, mated according to a minimal inbreeding scheme. The rabbits were free of all major rabbit pathogens and microbiological screening was conducted on a quarterly basis. Prior to delivery, the rabbits were housed in metal cages with an indirect palletted paper bedding system and trays were changed twice weekly.

Animal feed was obtained from Tillside Rabbit Stud (Penrith NSW) and rabbits were fed daily on a diet of young stock feed rabbit pellets, Lucerne cubes and tap water *ad libitum*. Diet was initially unrestricted, but due to aspiration in 2 cases, intake was limited to water for 2-hours before surgery.

Batches of five rabbits were transported to the Animal Care Facility at Westmead Hospital (Westmead NSW) and were between 8 and 10-weeks old on arrival depending on the experimental requirements. The animals were weighed on arrival and housed in individual steel cages (Batterie "P", Imbros Pty, Hobart, Tasmania). The internal dimension of each cage was 482 mm (depth), 580 mm (width) and 365 mm (height) providing a floor area of 2 × 800 cm². The cages were sited in an 8 foot (width) × 11 foot (length) × 9 foot (height) room, the temperature varied between 22°C and 24°C with 15 air changes per hour and a 12/12 light/dark cycle.

The general condition of the animals was assessed daily by an animal attendant. A veterinary surgeon was involved if there were any concerns and all interventions were documented.

2.6.2 Ethical Approval

Ethical approval was granted by the Animal Care & Ethics Committee, Western Sydney Area Health Service (Appendix 1). Experiments were conducted at the Animal Care Facility, Westmead Hospital and complied with the Australian code of practice for the care and use of animals for scientific purposes (National Health and Medical Research Council 1994). The experiments were performed under the direct supervision of the veterinary officer, Dr Margaret Ferrara who performed all *post mortem* examinations.

2.6.3 Laboratory Investigations

Haematological analysis was performed using a Technicon H3 automated laboratory analyser (Bayer AG, Leverkusen, Germany) and produced a FBC with a differential.

Biochemical analysis was performed using a Beckman Synchron CX5 auto analyser (Beckman Coulter Australia Pty., Ltd. Gladesville, NSW Australia). Analysis of sodium, potassium, chloride, bicarbonate and γ GT was performed using standard Beckman reagents. Analysis of urea, protein, calcium, magnesium, phosphate and alkaline phosphate was performed using reagents from Trace Scientific (Nobel Park Victoria Australia).

2.6.4 Anaesthesia

Anaesthesia was conducted by FM with the assistance of an experienced animal attendant (TJ). To minimise discomfort, all animals were premedicated 30-minutes before surgery with intra-muscular Ketamine, 10 mg/kg, (Parnell Laboratories Australia Pty Ltd, Alexandria, NSW), Xylazine, 4 mg/kg, (Ilium Xylazil-20, Troy Laboratories Pty Ltd, Smithfield, NSW, Australia), and Buprenorphine, 0.05 mg/kg (Reckitt & Coleman Products Ltd, Hull, UK).

General anaesthesia was induced via a face mask using an Enflurane (Abbott Australia Pty. Ltd. Botany NSW Australia) and Nitrous-oxide / Oxygen mixture. The

anaesthetic was performed without endotracheal intubation and was uncomplicated in the majority of cases. The rabbit was placed on a heating pad and covered with a polyethylene drape to keep it warm but core temperature was not monitored. After each anaesthetic, the animal was observed until alert and drinking before being returned to its cage.

2.6.5 Intravenous Access

An incision was made in the right groin, the superficial femoral neurovascular bundle was identified and the femoral vein was isolated. (Figure iii). A 20 gauge intravenous cannula (Terumo Corporation, Tokyo, Japan) was inserted and secured between 2 silk sutures providing a watertight seal.

The cannula was flushed to check for leaks and used to infuse either 50 ml adriamycin / cis-platinum (active) or 50 ml normal saline (control) over 2-hours using an IVAC[®] syringe pump. (ALARIS Medical Systems Australia, Pty. Ltd. Seven Hills NSW Australia) After an interval of 2-weeks, an identical infusion was delivered via a left groin incision using an identical approach.

In the dose response group, the animals were assessed on alternate days. In the main experimental group, the animals were assessed after each infusion, application of fixator and sacrifice. They were weighed and blood was obtained from an ear prick for full blood count (FBC), erythrocyte sedimentation rate (ESR), renal, hepatic and bone biochemistry.

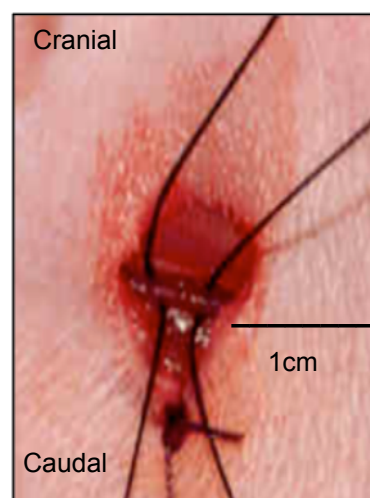


Figure iii Isolation of the Superficial Femoral Neurovascular Bundle

2.6.6 Chemotherapy Preparation and Delivery

The animal was evaluated 24-hours before infusion with particular reference to general condition, hydration and weight. The cytotoxic drugs were prepared under sterile conditions in the pharmacy of the Children's Hospital Westmead and delivered in pre-packed 50 ml syringes containing doses of cis-platinum and adriamycin in concentrations determined by the total body weight and phase of the experiment. These were stored overnight in a refrigerator and brought to room temperature 1-hour before infusion. Cytotoxic infusion was conducted over 2-hours under general anaesthesia using techniques described in section 2.6.4.

2.7 Dose Response Study

Seven juvenile (8-week) male NZW rabbits were infused with cis-platinum and adriamycin using decreasing drug combinations. The initial, maximum dose was suggested by the available literature and this was decreased until the experimental requirements were satisfied.

Each dose schedule was applied to a single rabbit to determine the dose which would be delivered to the active groups in the next phase of the experiment. This involved some risk, in that deterioration following the second infusion would require withdrawal of that group from further study, with a significant delay in completing the experiment. This risk was considered acceptable due to the time and budgetary constraints of the experiment and the estimations produced in the dose response study were robust and did not lead to withdrawal at any point.

Rabbit A	Cycle 1	6.5 mg/kg cis-platinum	6.5 mg/kg Adriamycin
	Cycle 2	6.5 mg/kg cis-platinum	6.5 mg/kg Adriamycin
Rabbit B	Cycle 1	4.5 mg/kg cis-platinum	4.5 mg/kg Adriamycin
	Cycle 2	4.5 mg/kg cis-platinum	4.5 mg/kg Adriamycin
Rabbit C	Cycle 1	3.0 mg/kg cis-platinum	3.0 mg/kg Adriamycin
	Cycle 2	3.0 mg/kg cis-platinum	3.0 mg/kg Adriamycin

Rabbit D	Cycle 1	2.0 mg/kg cis-platinum	2.0 mg/kg Adriamycin
	Cycle 2	2.0 mg/kg cis-platinum	2.0 mg/kg Adriamycin
Rabbit E	Cycle 1	1.0 mg/kg cis-platinum	1.0 mg/kg Adriamycin
	Cycle 2	1.0 mg/kg cis-platinum	1.0 mg/kg Adriamycin
Rabbit F	Cycle 1	1.0 mg/kg cis-platinum	2.0 mg/kg Adriamycin
	Cycle 2	1.0 mg/kg cis-platinum	4.0 mg/kg Adriamycin

Clinical parameters including body weight and haematological and biochemical indices were measured at intervals of approximately 48-hours.

The dose that produced a 5% reduction in percentage weight gain, without evidence of hepatic or renal failure was identified and used in the next component of the experiment to confirm that the model was robust

The following section contains results from the dose response study presented as data from individual animals. Descriptive statistics from the main experimental groups are also presented to validate the animal model and demonstrate its suitability for use in the investigation of the characteristics of regenerate bone produced by distraction osteogenesis.

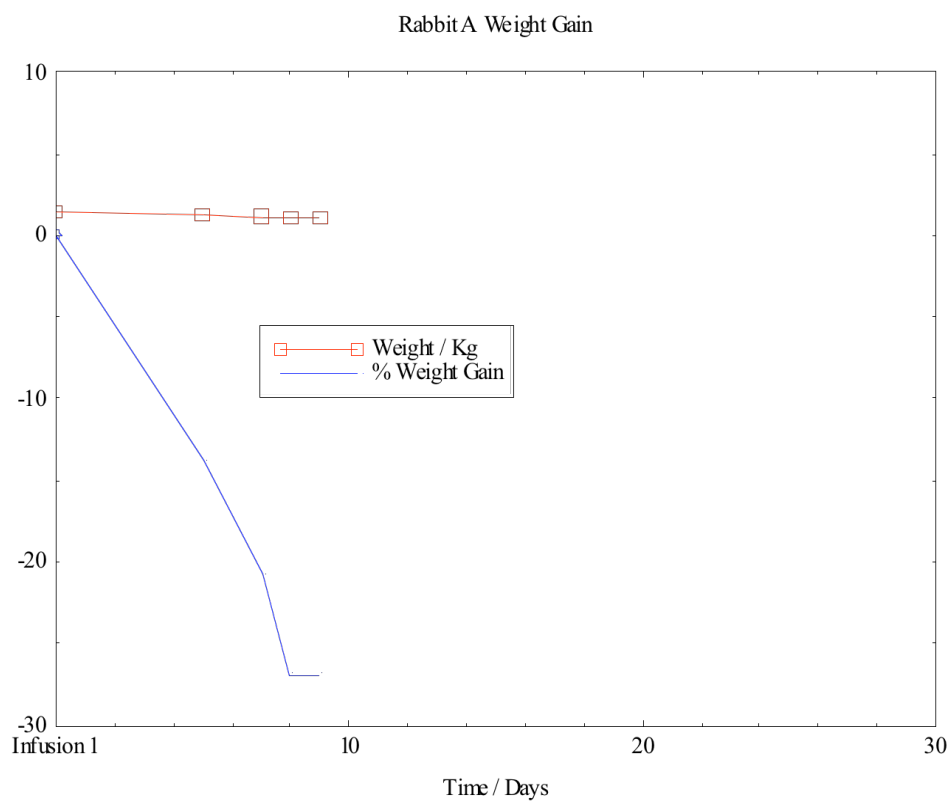
2.8 Dose Response Study Results

2.8.1 Rabbit A

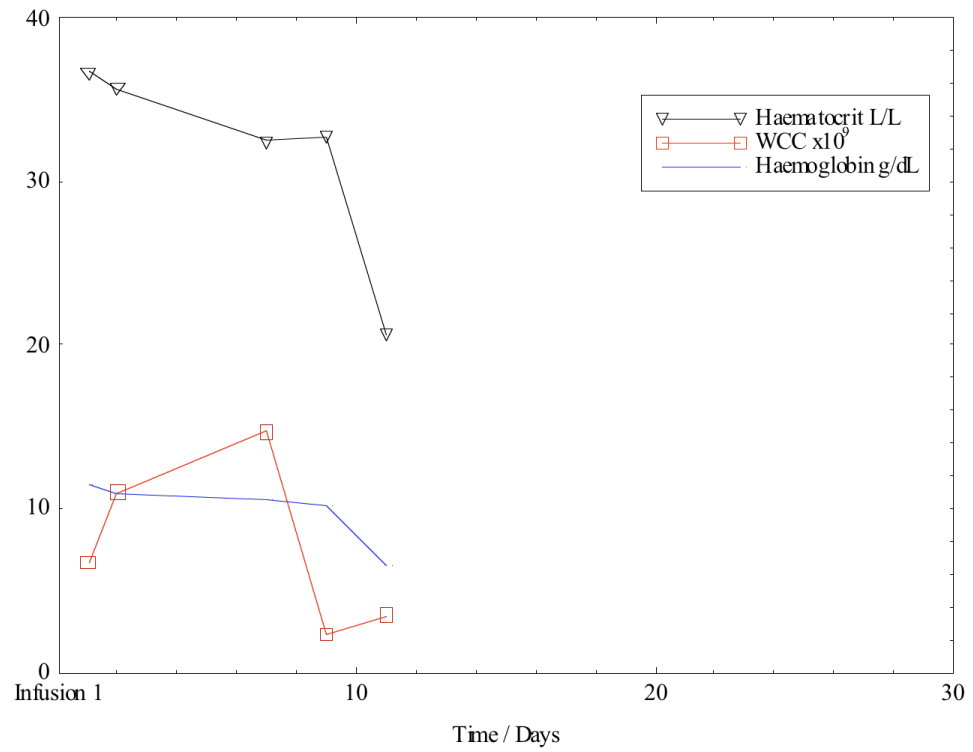
Cycle 1	6.5 mg/kg cis-platinum	6.5 mg/kg adriamycin
Cycle 2	6.5 mg/kg cis-platinum	6.5 mg/kg adriamycin

On day 8, there was an obvious loss of appetite and a further deterioration leading to withdrawal of the animal and euthanasia on day 11.

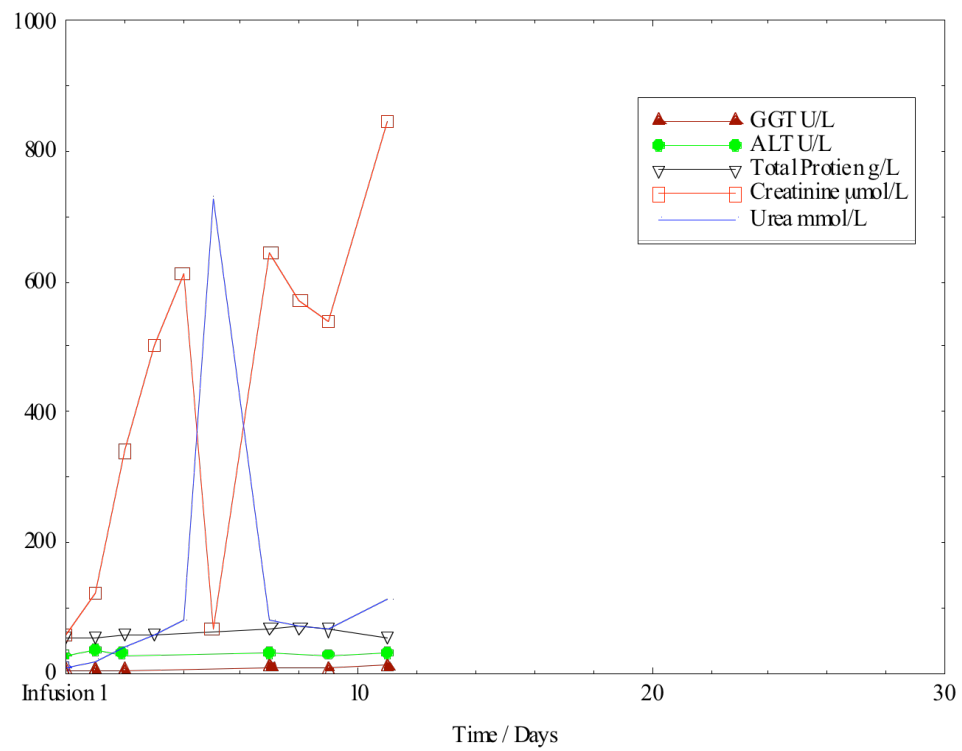
Post mortem examination demonstrated pericardial blood stained fluid with enlargement of both kidneys. The renal parenchyma was granular with an increase in peri-renal fat.



Rabbit A Haematology



Rabbit A Biochemistry

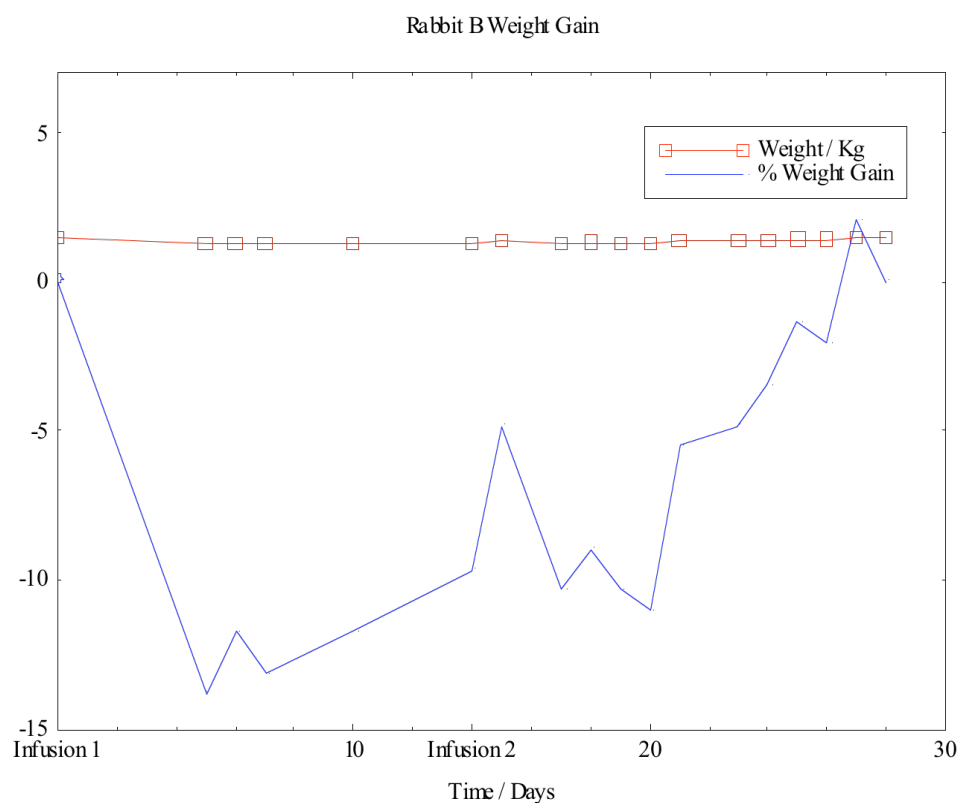


2.8.2 Rabbit B

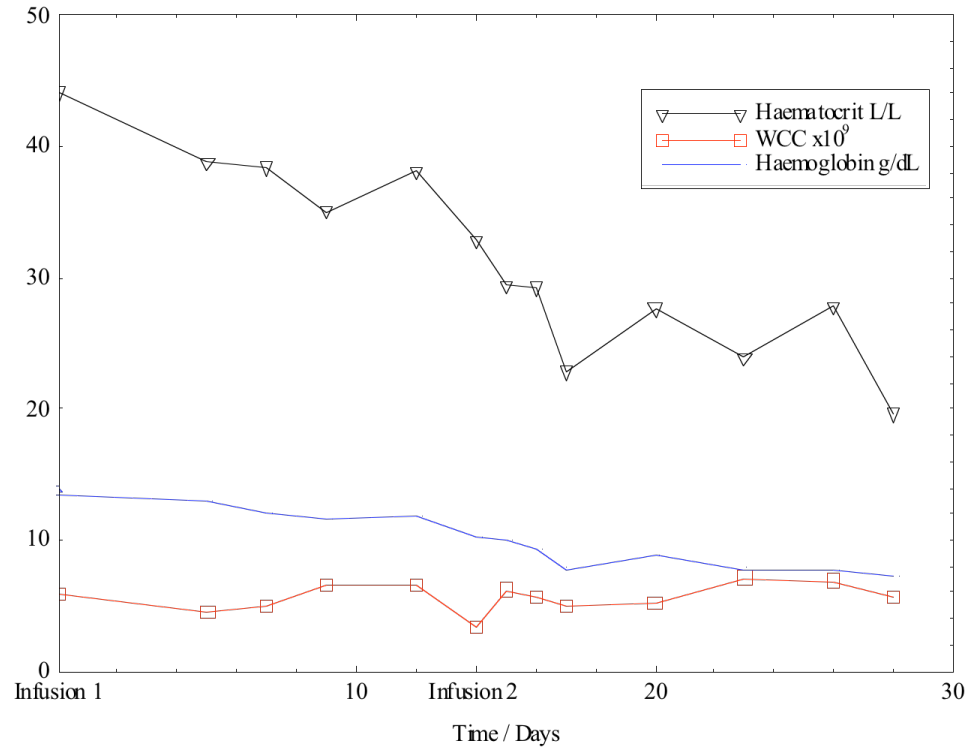
Cycle 1	4.5 mg/kg cis-platinum	4.5 mg/kg adriamycin
Cycle 2	4.5 mg/kg cis-platinum	4.5 mg/kg adriamycin

On day 17, the animal was not eating or drinking and required subcutaneous normal saline injections over a 4-day period. This produced a full recovery and the animal was in good condition at the time of sacrifice on day 28.

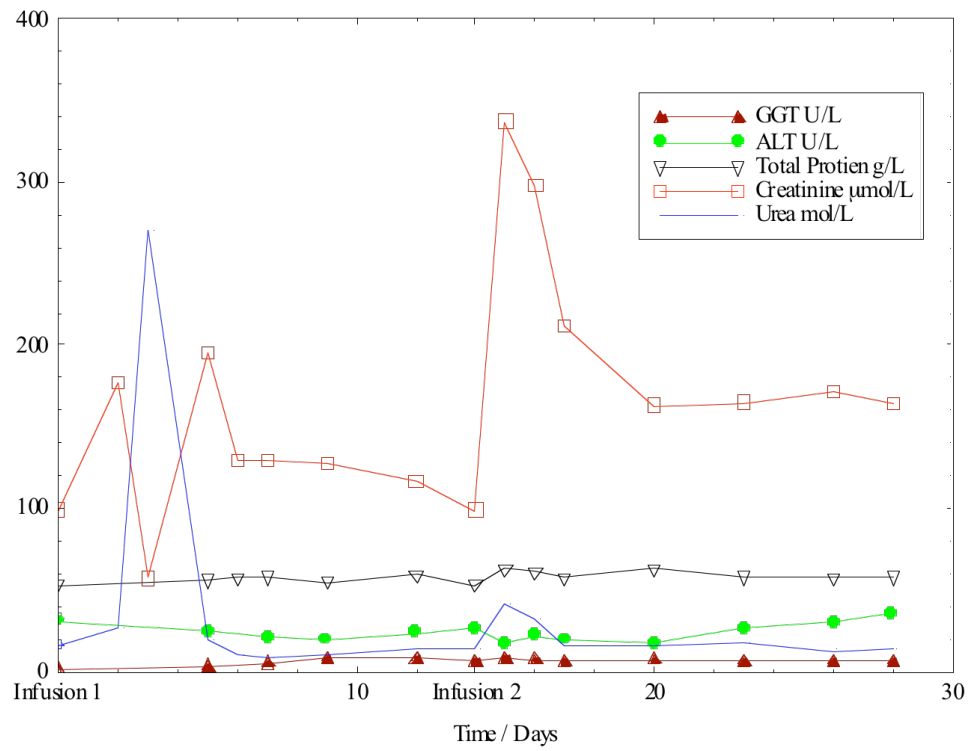
Post mortem examination demonstrated a macroscopically normal heart and liver with minimally enlarged kidneys, which were morphologically normal.



Rabbit B Haematology



Rabbit B Biochemistry



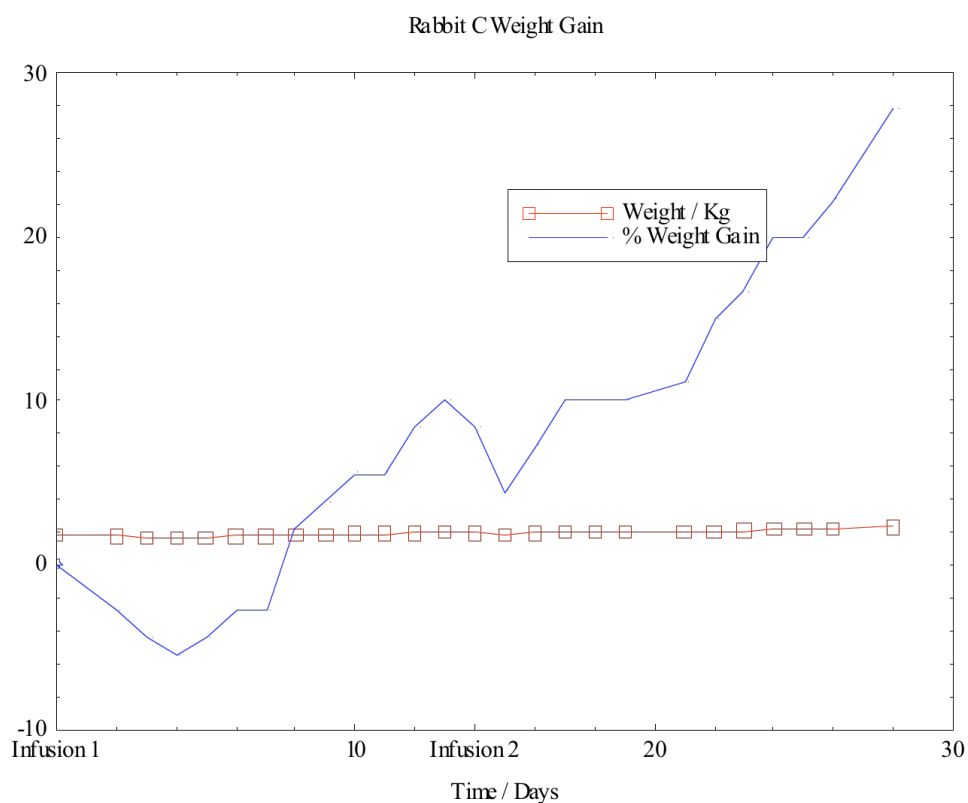
2.8.3 Rabbit C

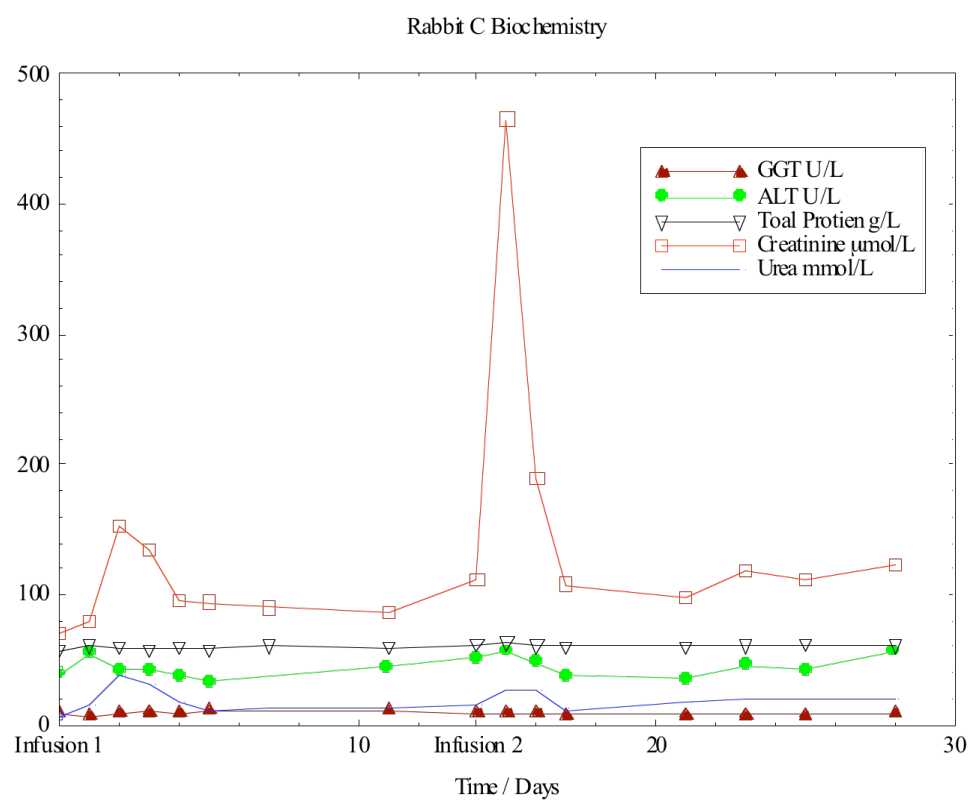
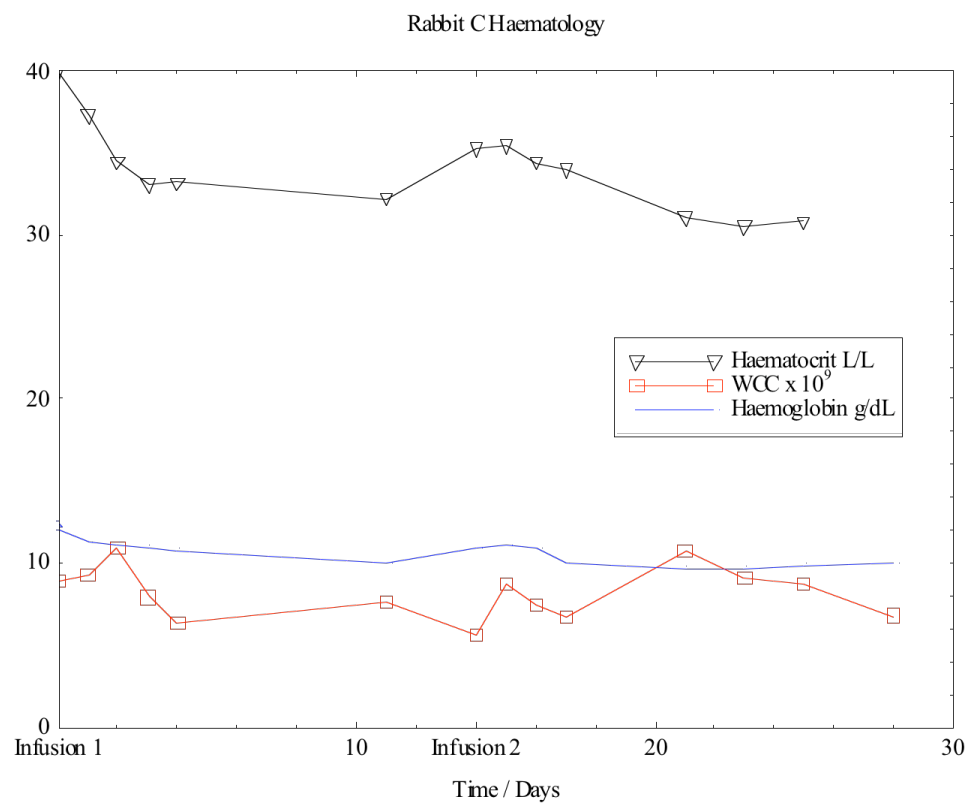
Cycle 1	2.0 mg/kg cis-platinum	2.0 mg/kg adriamycin
Cycle 2	2.0 mg/kg cis-platinum	2.0 mg/kg adriamycin

A superficial wound dehiscence (3 mm) occurred on day 3. This did not require treatment and as the animal was in good condition, the experiment continued and healing was complete by day 14.

The left inguinal cannulation and infusion were uncomplicated and the animal remained in a good clinical condition until the time of sacrifice on day-28.

Post mortem examination demonstrated a macroscopically normal heart, liver and kidneys



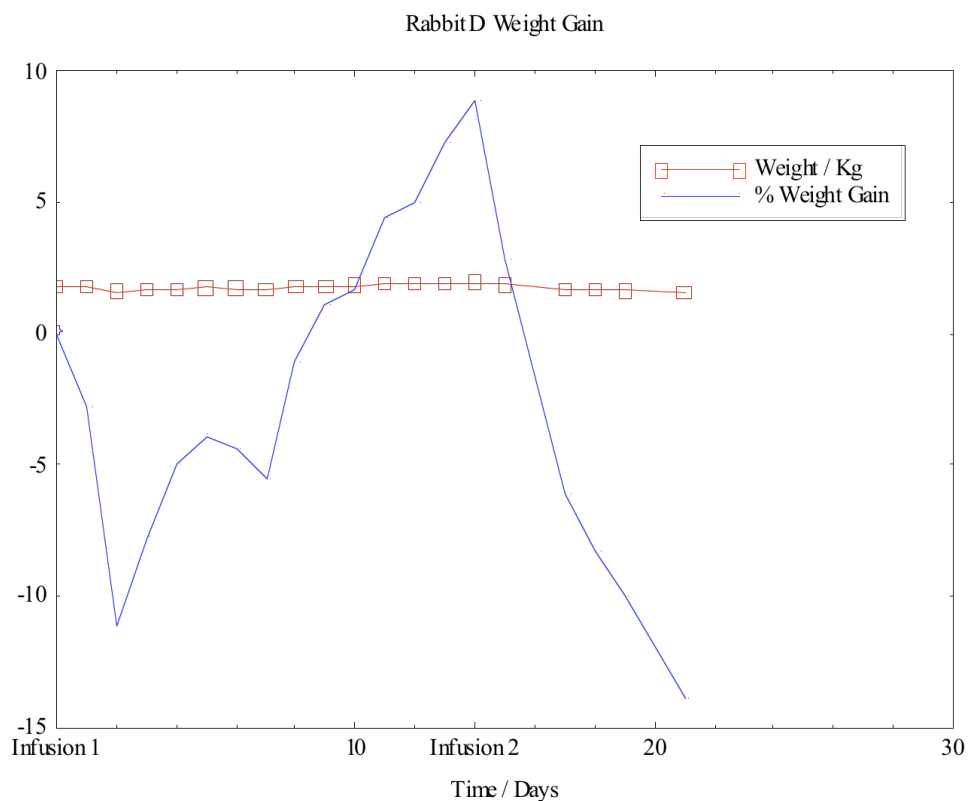


2.8.4 Rabbit D

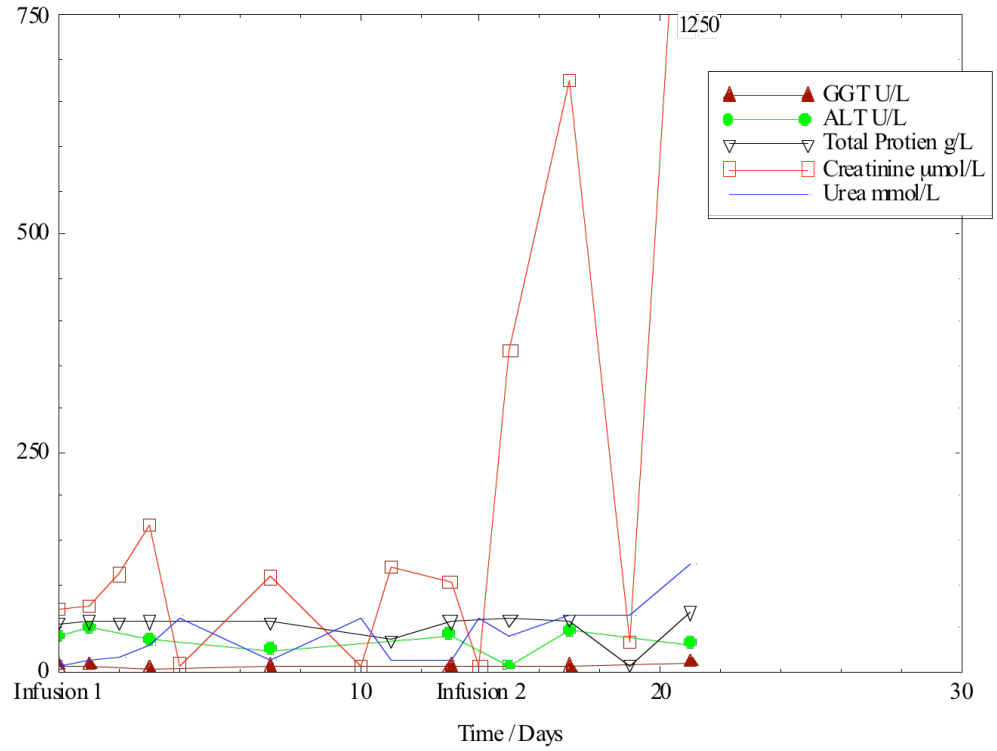
Cycle 1	3.0 mg/kg cis-platinum	3.0 mg/kg adriamycin
Cycle 2	3.0 mg/kg cis-platinum	3.0 mg/kg adriamycin

Each cannulation and infusion was performed without incident. On day 19, there was a profound deterioration in general condition leading to death on day 21.

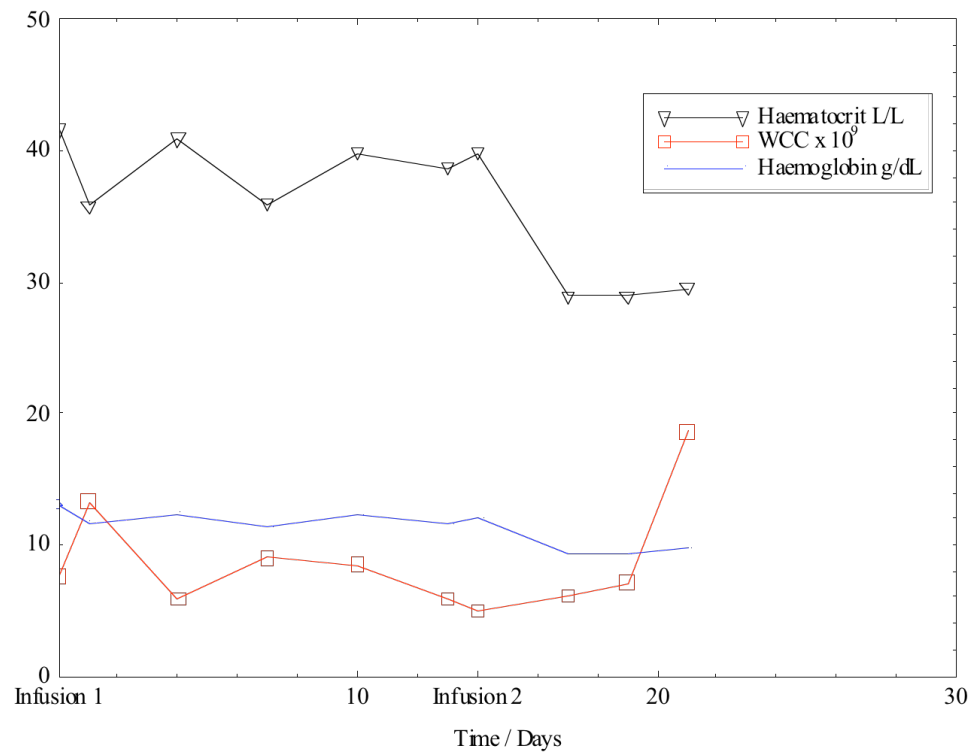
Post mortem examination demonstrated macroscopically normal heart and liver. There was moderate enlargement of the kidneys with a granular parenchymal texture and increase in peri-renal fat.



Rabbit D Biochemistry



Rabbit D Haematology



2.8.5 Rabbit E

Cycle 1 1.0 mg/kg cis-platinum 1.0 mg/kg adriamycin

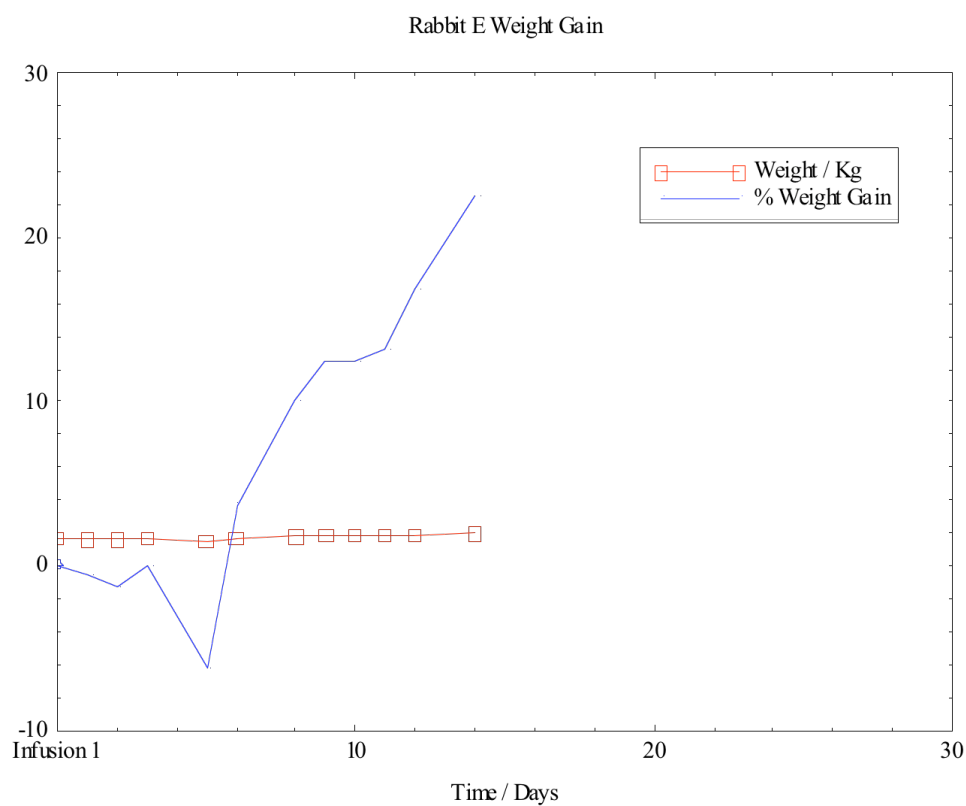
Cycle 2 1.0 mg/kg cis-platinum 1.0 mg/kg adriamycin

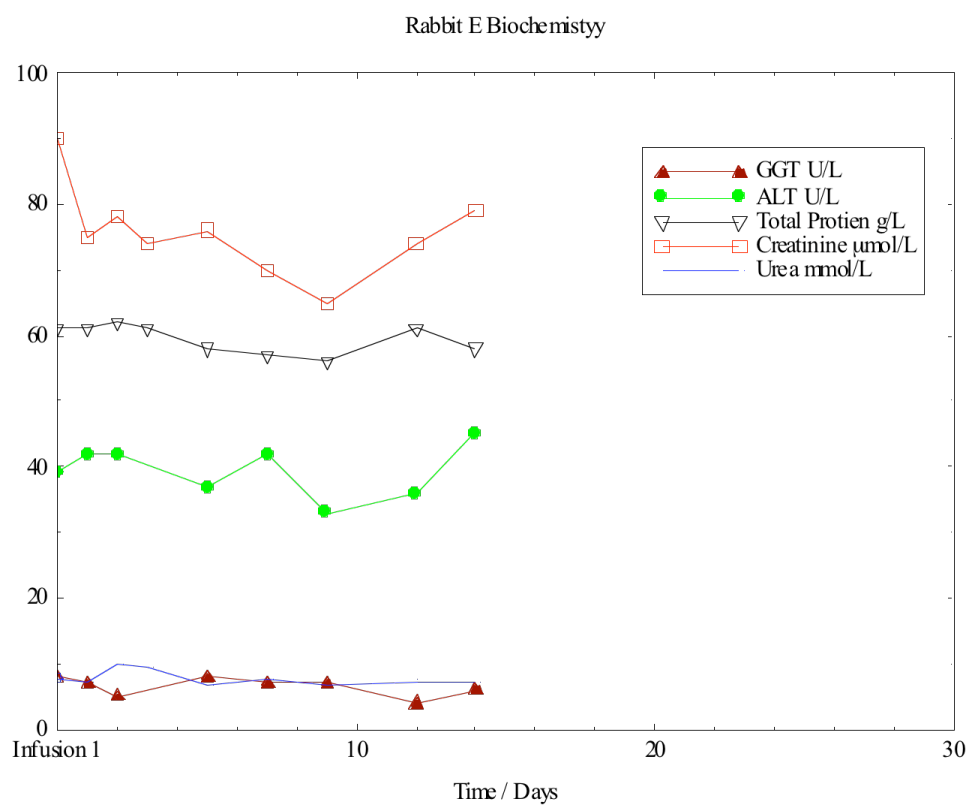
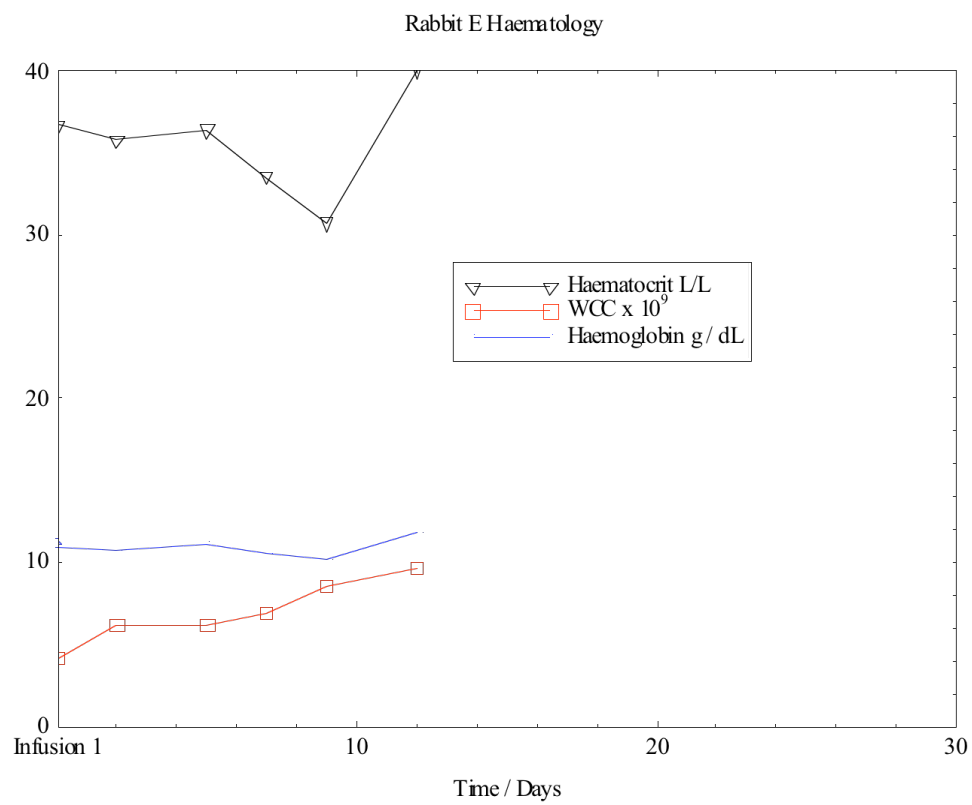
The right inguinal cannulation and infusion were conducted without complication.

The general condition was satisfactory following cannulation and the wound healed without incident.

At the beginning of the second infusion, the animal sustained a fatal cardiac arrest.

Post mortem examination demonstrated normal a heart, liver, kidneys. The lungs were normal with no evidence of aspiration and the presumed cause of death was an air embolism secondary to cannulation.



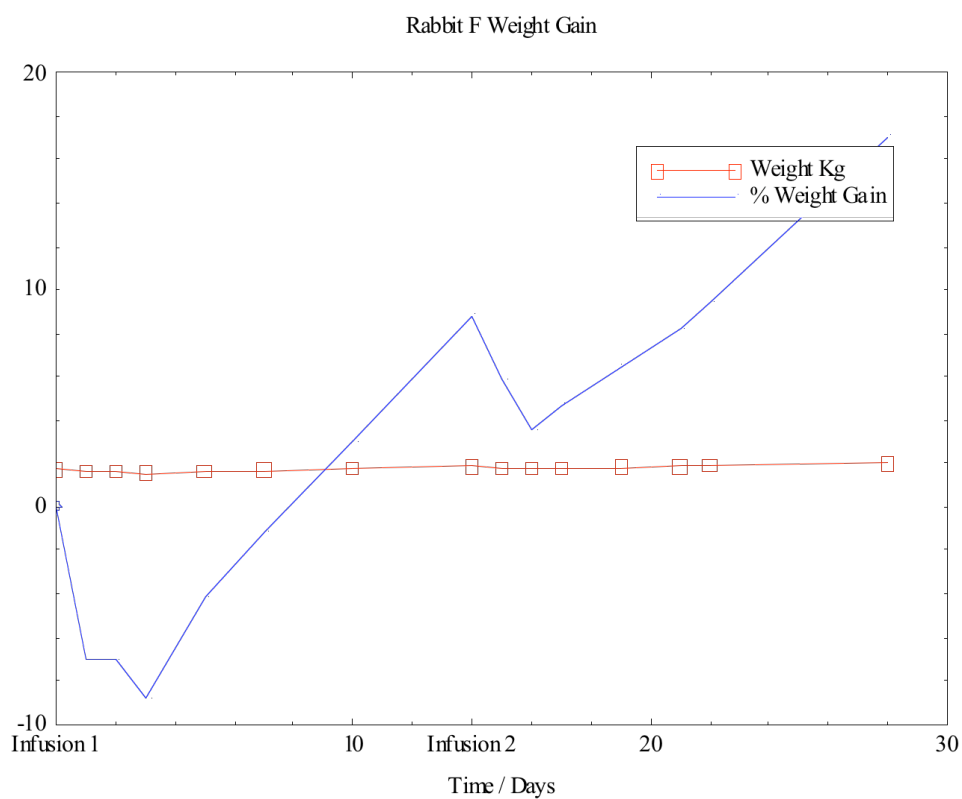


2.8.6 Rabbit F

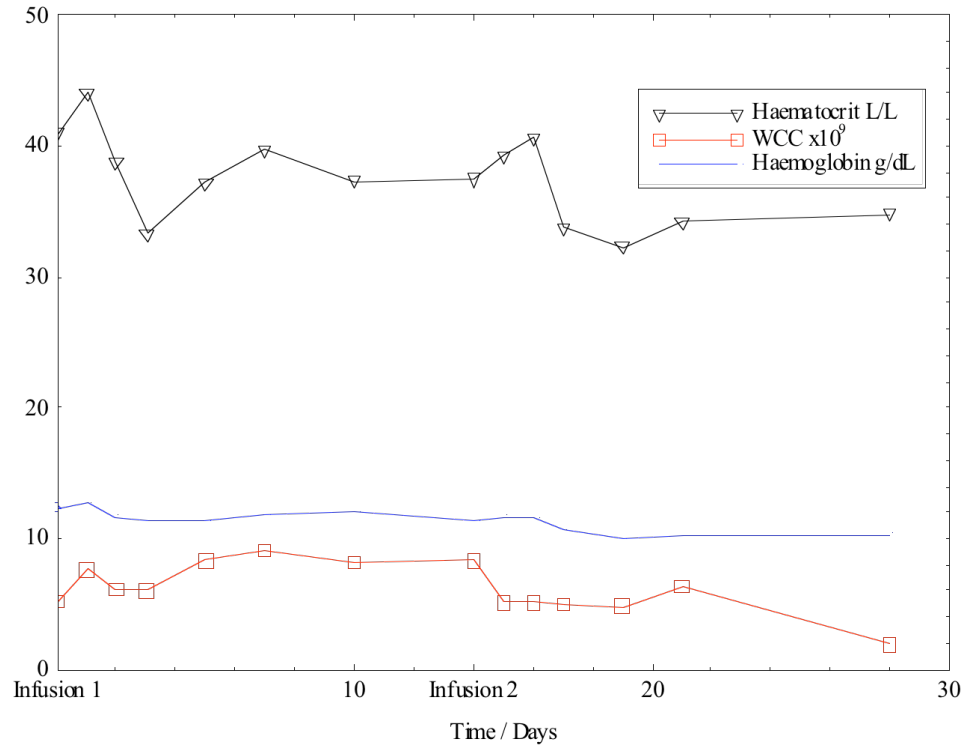
Cycle 1	1.0 mg/kg cis-platinum	2.0 mg/kg adriamycin
Cycle 2	1.0 mg/kg cis-platinum	4.0 mg/kg adriamycin

The initial groin exposure, cannulation and infusion were uncomplicated with rapid wound healing. The second groin exposure cannulation and infusion was also uncomplicated with rapid wound healing. The general condition remained satisfactory until the time of sacrifice at day 28.

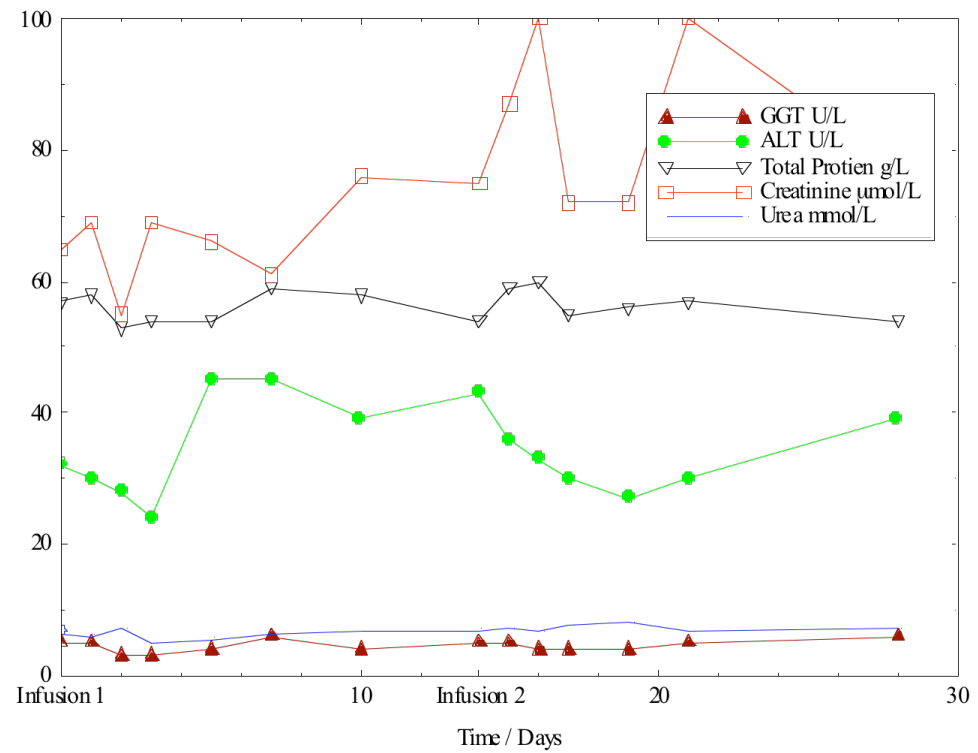
Post mortem examination demonstrated a macroscopically normal heart, liver and kidneys. The femoral and external iliac veins and inferior vena cava were patent without any complication related to cannulation.



Rabbit F Haematology



Rabbit F Biochemistry



2.9 Summary of Dose Response Study

Doses of adriamycin and cis-platinum in excess of 3 mg/kg produced deterioration in general condition and renal function and at 6.5 mg/kg led to death 8-days after the initial infusion.

A dose of 4.5mg/kg caused decline in renal function after each infusion, which initially recovered but deteriorated after the second infusion and had not recovered at the end of the period of observation.

At 3 mg/kg there was deterioration in general condition leading to death at day 19, suggesting an idiosyncratic sensitivity to these drugs in some animals.

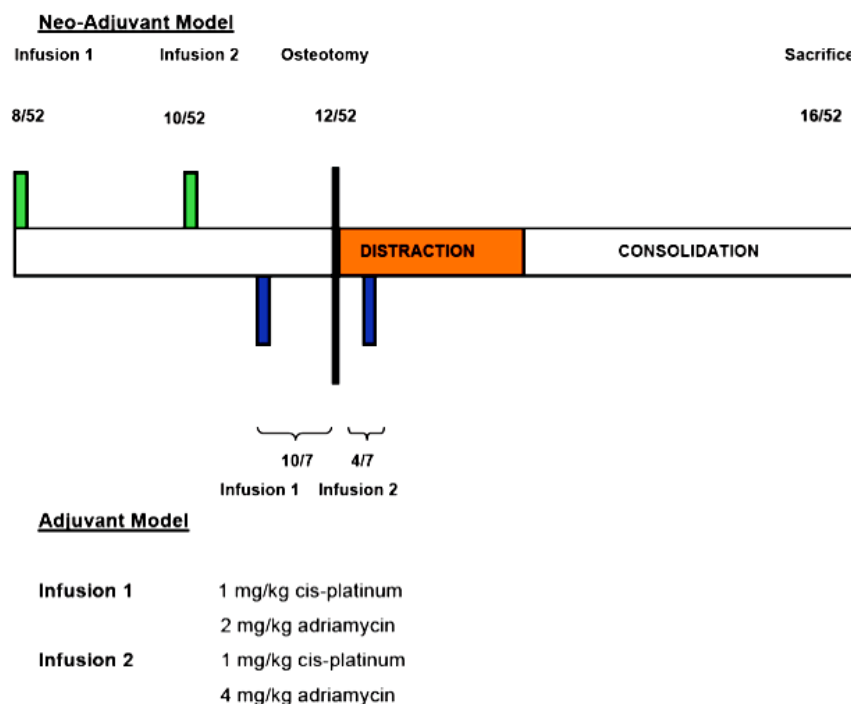
A more modest increase in creatinine was observed following the first infusion of 2 mg/kg but there was a permanent increase in creatinine after the second infusion suggesting a cumulative effect on renal function even at this low dose. This was considered to be due to the effect of cis-platinunum, which has been previously demonstrated to be nephrotoxic in this species (Frank et al., 1989) and suggested that the dose of cis-platinum should be limited to 1mg/kg.

In the animal that received 1mg/kg cis-platinum and adriamycin there was a modest effect on body weight which recovered by day 6. This suggested that the cytotoxic effect was insufficient to produce a clinically relevant response, and would not mirror the effect that would be expected in a human patient receiving therapeutic doses of these agents. Death occurred as a result of experimental error but there was no biochemical evidence of renal impairment during the period of study.

The dosage schedule in the distraction osteogenesis experiment used an initial infusion of 1mg/kg cis-platinum to protect renal function and 2mg/kg adriamycin. If the initial infusion demonstrated idiosyncratic sensitivity, the animal could be withdrawn with sufficient time to substitute and maintain experimental numbers. If the condition of the animal was satisfactory it would allow progression to a second dose of 4mg/kg and this was possible in all experimental animals.

The timing of drug administration was also investigated and the schedules were broadly defined as neo- adjuvant if drugs were given before surgery and neo- adjuvant if drugs were given after surgery. Both strategies are used in the management of osteogenic sarcoma and the model attempted to mimic the COSS (Link et al., 1991, Link et al., 1986) and EOI (Bramwell et al., 1992, Souhami et al., 1997) approach to this clinical problem.

This formed the basis of the cytotoxic chemotherapy schedule for the distraction osteogenesis experiment. The drugs were given 4 and 2 weeks before osteotomy and distraction (Neo-adjuvant Group) or one cycle 10 days before and 4 days after osteotomy and distraction (Adjuvant Group).



The next section reports the results that were used to validate the model and confirm that the prerequisites defined in previous paragraphs were satisfied.

2.10 Laboratory Parameters

2.10.1 Neo-adjuvant Group Haemoglobin (g/dl)

		Mean	σ
Infusion 1	(Control)	11.69	1.16
	(Chemotherapy)	12.84	0.76
Infusion 2	(Control)	12.25	0.98
	(Chemotherapy)	12.38	0.80
Osteotomy	(Control)	12.96	0.82
	(Chemotherapy)	11.28	0.71
Sacrifice	(Control)	12.79	1.23
	(Chemotherapy)	12.96	0.76

Infusion 1

$p = < 0.01$

95% confidence interval for difference between means = -1.98 to -0.3

Power (for 5% significance) = 97.22%

Infusion 2

$p = 0.75$

95% confidence interval for difference between means = -0.92 to 0.67

Power (for 5% significance) = 7.04%

Osteotomy

$p = < 0.01$

95% confidence interval for difference between means = 0.94 to 2.41

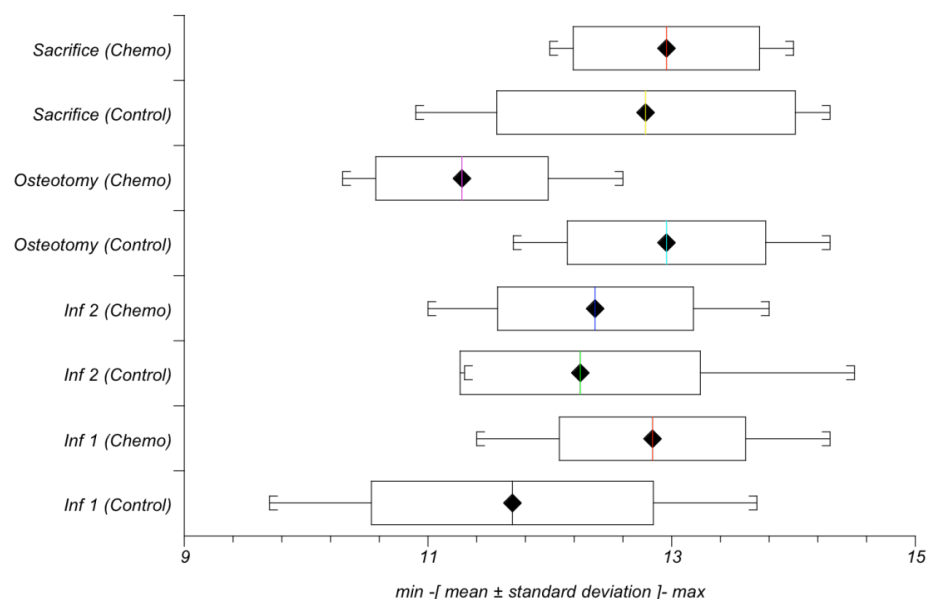
Power (for 5% significance) > 99.99%

Sacrifice

$p = 0.76$

95% confidence interval for difference between means = -1.31 to 0.97

Power (for 5% significance) = 7.18%



2.10.2 Adjuvant Group Haemoglobin (g/dl)

		Mean	σ
Infusion 1	(Control)	13.31	0.87
	(Chemotherapy)	13.03	0.88
Osteotomy	(Control)	13.08	0.84
	(Chemotherapy)	12.13	0.71
Infusion 2	(Control)	13.40	0.26
	(Chemotherapy)	10.19	0.56
Sacrifice	(Control)	12.44	1.04
	(Chemotherapy)	11.79	0.78

Infusion 1

$p = 0.44$

95% confidence interval for difference between means = -0.48 to 1.04

Power (for 5% significance) = 17.52%

Osteotomy

$p = 0.01$

95% confidence interval for difference between means = 0.21 to 1.67

Power (for 5% significance) = 91.56%

Infusion 2

$p < 0.01$

95% confidence interval for difference between means = 2.42 to 4.01

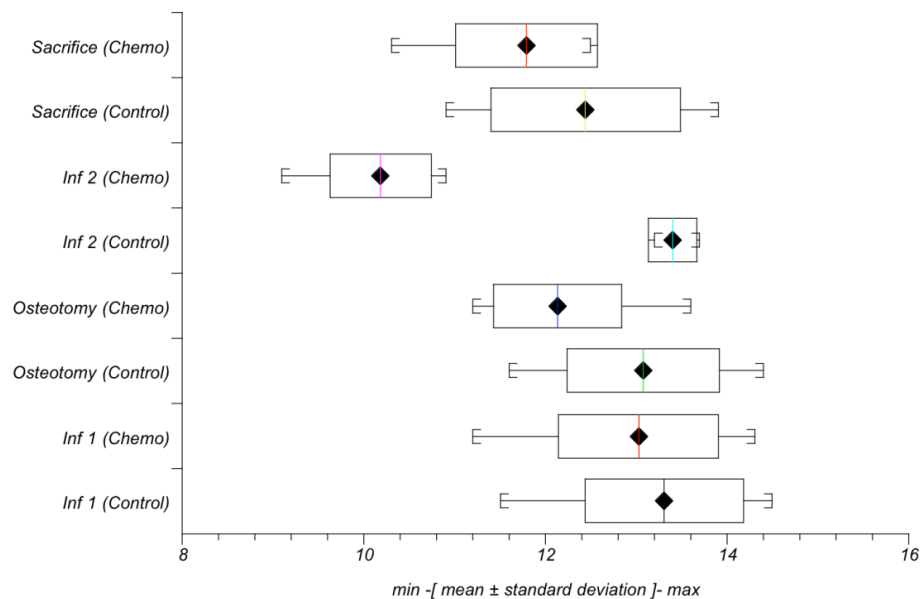
Power (for 5% significance) > 99.99%

Sacrifice

$p = 0.15$

95% confidence interval for difference between means = -0.26 to 1.56

Power (for 5% significance) = 44.9%



2.10.3 Neo-adjuvant Group Haematocrit (L/L)

		Mean	σ
Infusion 1	(Control)	42.67	11.77
	(Chemotherapy)	42.62	2.16
Infusion 2	(Control)	42.21	7.70
	(Chemotherapy)	40.50	2.91
Osteotomy	(Control)	43.42	7.78
	(Chemotherapy)	37.52	2.21
Sacrifice	(Control)	42.69	9.05
	(Chemotherapy)	44.40	5.53

Infusion 1

p = 0.99 (F test significant)

95% confidence interval for difference between means = -7.11 to 7.21

Power (for 5% significance) = 2.57%

Infusion 2

p = 0.52 (F test significant)

95% confidence interval for difference between means = -3.66 to 7.08

Power (for 5% significance) = 7.65%

Osteotomy

p = 0.06 (F test significant)

95% confidence interval for difference between means = 0.23 to 11.57

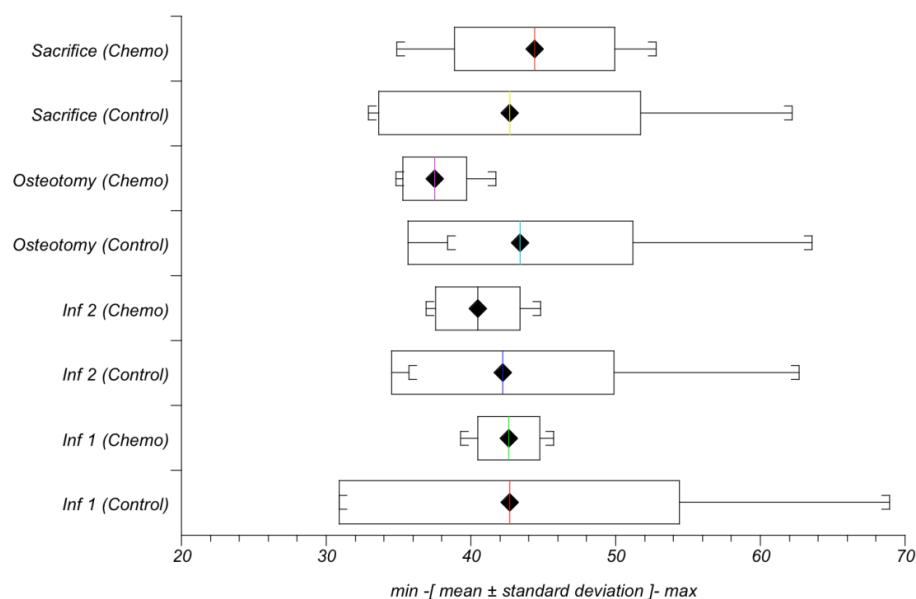
Power (for 5% significance) = 47.7%

Sacrifice

p = 0.67

95% confidence interval for difference between means = -10.08 to 6.65

Power (for 5% significance) = 9.24%



2.10.4 Adjuvant Group Haematocrit (L/L)

		Mean	σ
Infusion 1	(Control)	41.27	2.62
	(Chemotherapy)	42.53	2.49
Osteotomy	(Control)	42.16	2.90
	(Chemotherapy)	39.01	2.00
Infusion 2	(Control)	42.93	0.81
	(Chemotherapy)	39.01	2.00
Sacrifice	(Control)	37.06	2.26
	(Chemotherapy)	39.89	2.17

Infusion 1

$p = 0.25$

95% confidence interval for difference between means = -3.47 to 0.96

Power (for 5% significance) = 34.01%

Osteotomy

$p = >0.01$

95% confidence interval for difference between means = 0.86 to 5.45

Power (for 5% significance) = 94.36%

Infusion

$p = >0.01$

95% confidence interval for difference between means = 1.32 to 6.53

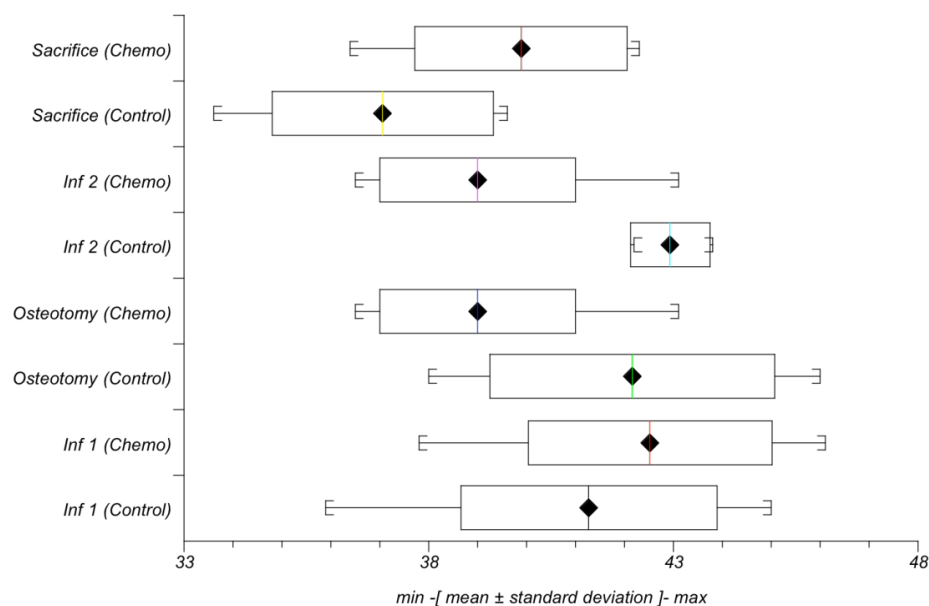
Power (for 5% significance) = 92.53%

Sacrifice

$p = 0.02$

95% confidence interval for difference between means = -5.09 to -0.58

Power (for 5% significance) = 89.59%



2.10.5 Neo-adjuvant Group Leucocyte Count (x109)

		Mean	σ
Infusion 1	(Control)	4.53	1.73
	(Chemotherapy)	4.36	0.82
Infusion 2	(Control)	5.15	1.71
	(Chemotherapy)	4.58	1.04
Osteotomy	(Control)	4.50	1.78
	(Chemotherapy)	3.09	1.89
Sacrifice	(Control)	8.36	3.96
	(Chemotherapy)	8.86	4.57

Infusion 1

p = 0.77 (F test significant)

95% confidence interval for difference between means = -0.98 to 1.31

Power (for 5% significance) = 4.38%

Infusion 2

p = 0.34

95% confidence interval for difference between means = -0.66 to 1.81

Power (for 5% significance) = 23.91%

Osteotomy

p = 0.11

95% confidence interval for difference between means = -0.37 to 3.19

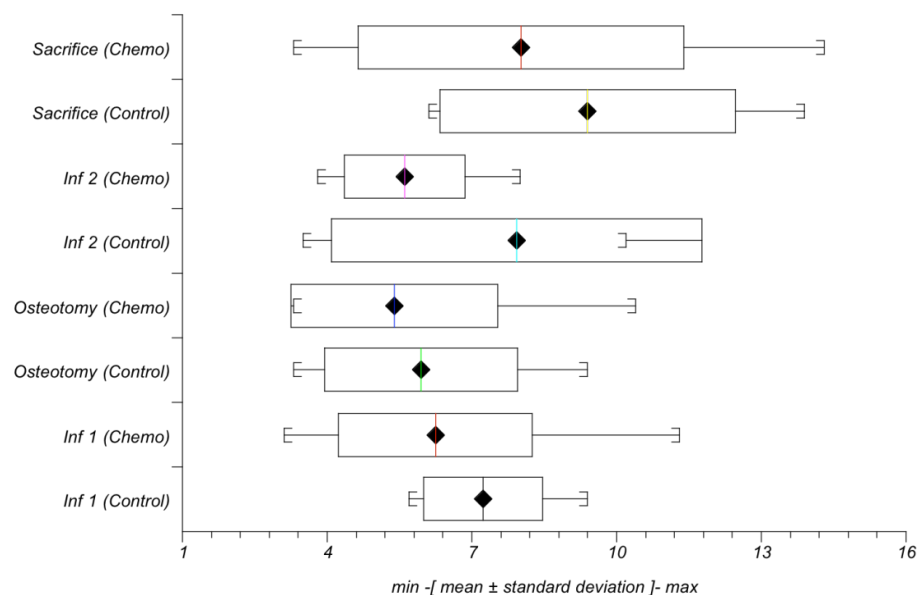
Power (for 5% significance) = 58.16%

Sacrifice

p = 0.82

95% confidence interval for difference between means = -5.08 to 4.07

Power (for 5% significance) = 6.19%



2.10.6 Adjuvant Group Leucocyte Count (x10⁹)

		Mean	σ
Infusion 1	(Control)	7.23	1.23
	(Chemotherapy)	6.24	2.02
Osteotomy	(Control)	5.95	2.00
	(Chemotherapy)	5.39	2.14
Infusion 2	(Control)	7.93	3.84
	(Chemotherapy)	5.61	1.25
Sacrifice	(Control)	9.40	3.07
	(Chemotherapy)	8.02	3.37

Infusion 1

p = 0.18

95% confidence interval for difference between means = -0.48 to 2.45

Power (for 5% significance) = 45.39%

Osteotomy

p = 0.58

95% confidence interval for difference between means = -1.45 to 2.56

Power (for 5% significance) = 10.97%

Infusion 2

p = 0.41 (F test significant)

95% confidence interval for difference between means = -2.79 to 7.43

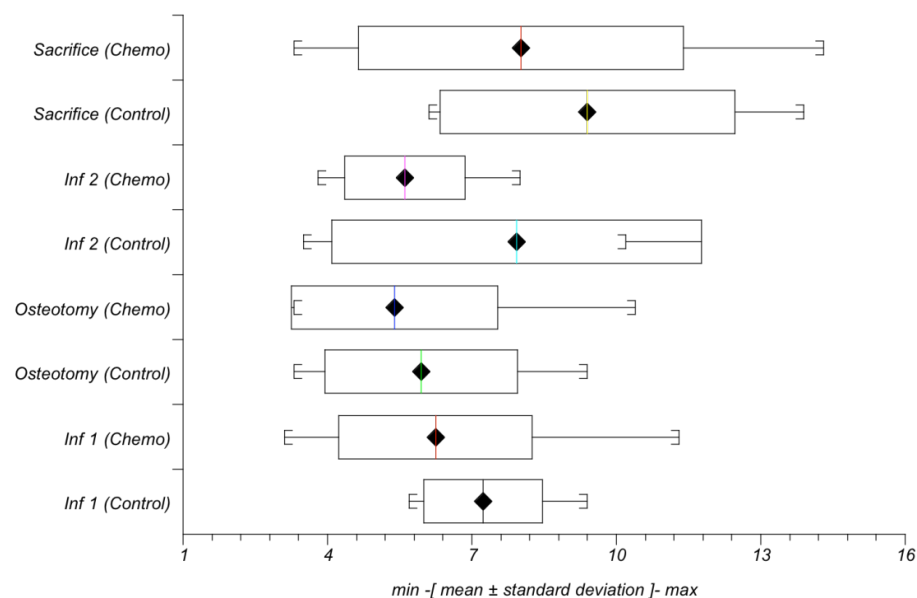
Power (for 5% significance) = 4.39%

Sacrifice

p = 0.39

95% confidence interval for difference between means = -1.96 to 4.72

Power (for 5% significance) = 18.27%



2.10.7 Neo-adjuvant Group Urea (mmol/L)

		Mean	σ
Infusion 1	(Control)	6.35	0.95
	(Chemotherapy)	6.31	0.69
Infusion 2	(Control)	6.32	0.85
	(Chemotherapy)	7.43	0.74
Osteotomy	(Control)	7.23	0.78
	(Chemotherapy)	7.87	1.57
Sacrifice	(Control)	7.00	1.28
	(Chemotherapy)	6.97	1.46

Infusion 1

$p = 0.90$

95% confidence interval for difference between means = -0.66 to 0.75

Power (for 5% significance) = 5.31%

Infusion 2

$p = > 0.01$

95% confidence interval for difference between means = -1.81 to -0.40

Power (for 5% significance) = 98.71%

Osteotomy

$p = 0.28$ (F test significant)

95% confidence interval for difference between means = -1.76 to 0.48

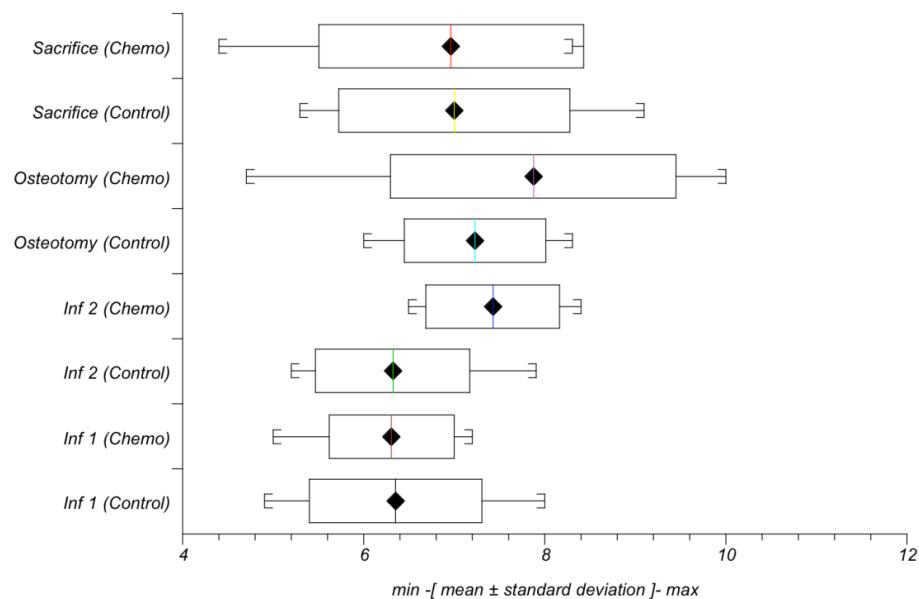
Power (for 5% significance) = 18.33%

Sacrifice

$p = 0.96$

95% confidence interval for difference between means = -1.46 to 1.52

Power (for 5% significance) = 5.06%



2.10.8 Adjuvant Group Urea (mmol/L)

		Mean	σ
Infusion 1	(Control)	4.62	0.71
	(Chemotherapy)	5.73	0.71
Osteotomy	(Control)	4.78	0.77
	(Chemotherapy)	6.49	2.04
Infusion 2	(Control)	5.42	0.48
	(Chemotherapy)	6.62	1.18
Sacrifice	(Control)	5.38	0.59
	(Chemotherapy)	6.31	1.83

Infusion 1

$p = >0.01$

95% confidence interval for difference between means = -1.72 to -0.49

Power (for 5% significance) = 99.84%

Osteotomy

$p = 0.02$ (F test significant)

95% confidence interval for difference between means = -3.04 to -0.38

Power (for 5% significance) = 70.56%

Infusion 2

$p = < 0.05$

95% confidence interval for difference between means = -2.39 to -0.006

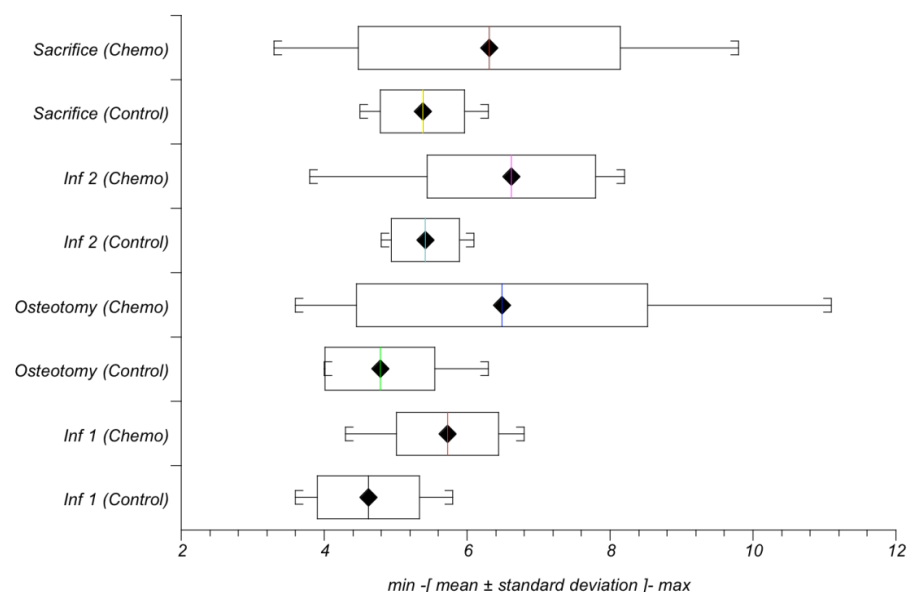
Power (for 5% significance) = 68.62%

Sacrifice

$p = 0.14$ (F test significant)

95% confidence interval for difference between means = -2.16 to 0.30

Power (for 5% significance) = 28.51%



2.10.9 Neo-adjuvant Group Creatinine ($\mu\text{mol/L}$)

		Mean	σ
Infusion 1	(Control)	75.58	23.20
	(Chemotherapy)	68.25	8.47
Infusion 2	(Control)	73.50	11.15
	(Chemotherapy)	76.33	8.44
Osteotomy	(Control)	81.10	9.39
	(Chemotherapy)	85.64	17.40
Sacrifice	(Control)	87.11	10.03
	(Chemotherapy)	88.33	13.16

Infusion 1

$p = 0.32$ (F test significant)

95% confidence interval for difference between means = -7.45 to 22.12

Power (for 5% significance) = 14.13%

Infusion 2

$p = 0.51$

95% confidence interval for difference between means = -11.54 to 5.87

Power (for 5% significance) = 14.01%

Osteotomy

$p = 0.47$

95% confidence interval for difference between means = -17.51 to 8.43

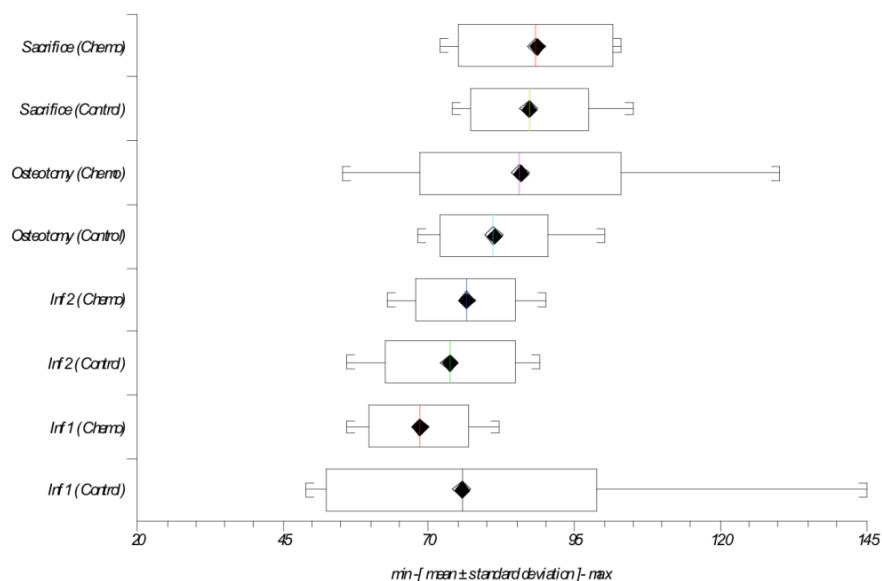
Power (for 5% significance) = 15.88%

Sacrifice

$p = 0.84$

95% confidence interval for difference between means = -14.13 to 11.68

Power (for 5% significance) = 5.96%



2.10.10 Adjuvant Group Creatinine ($\mu\text{mol/L}$)

		Mean	σ
Infusion 1	(Control)	67.82	4.09
	(Chemotherapy)	71.50	8.57
Osteotomy	(Control)	67.90	7.09
	(Chemotherapy)	87.50	21.32
Infusion 2	(Control)	70.50	5.96
	(Chemotherapy)	82.73	12.05
Sacrifice	(Control)	88.33	12.03
	(Chemotherapy)	91.36	8.74

Infusion 1

$p = 0.20$ (F test significant)

95% confidence interval for difference between means = -9.43 to 2.07

Power (for 5% significance) = 22.14%

Osteotomy

$p = < 0.01$ (F test significant)

95% confidence interval for difference between means = -33.27 to -5.93

Power (for 5% significance) = 79.36%

Infusion 2

$p = 0.04$

95% confidence interval for difference between means = -23.50 to -0.95

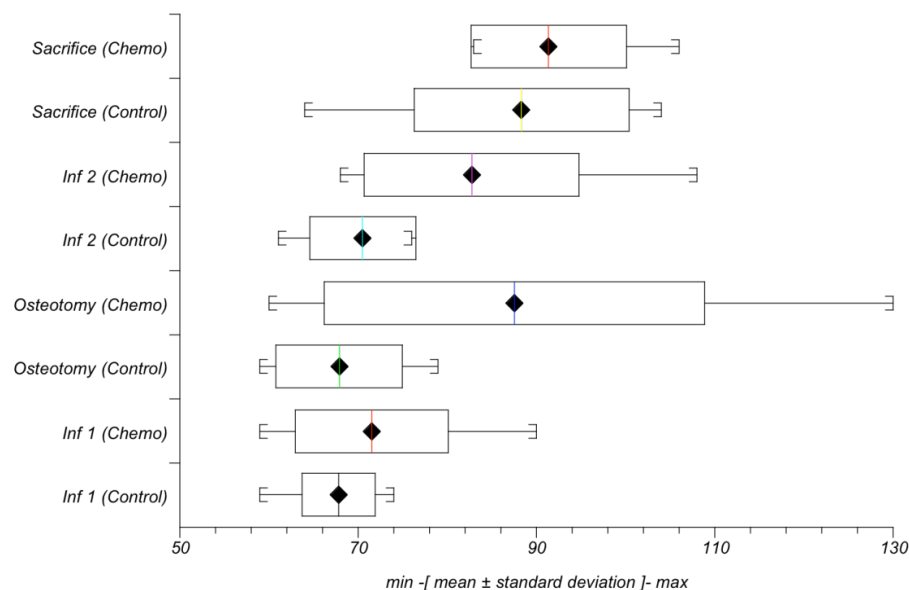
Power (for 5% significance) = 77.41%

Sacrifice

$p = 0.52$

95% confidence interval for difference between means = -12.79 to 6.73

Power (for 5% significance) = 13.12%



2.10.11 Neo-adjuvant Group Total Protein (g/L)

		Mean	σ
Infusion 1	(Control)	55.00	5.15
	(Chemotherapy)	56.25	3.60
Infusion 2	(Control)	55.90	3.57
	(Chemotherapy)	54.42	3.68
Osteotomy	(Control)	58.40	4.22
	(Chemotherapy)	52.91	2.30
Sacrifice	(Control)	59.80	7.18
	(Chemotherapy)	56.17	3.92

Infusion 1

$p = 0.5$

95% confidence interval for difference between means = -5.01 to 2.51

Power (for 5% significance) = 15.23%

Infusion 2

$p = 0.35$

95% confidence interval for difference between means = -1.74 to 4.71

Power (for 5% significance) = 23.47%

Osteotomy

$p = < 0.01$

95% confidence interval for difference between means = 2.43 to 8.56

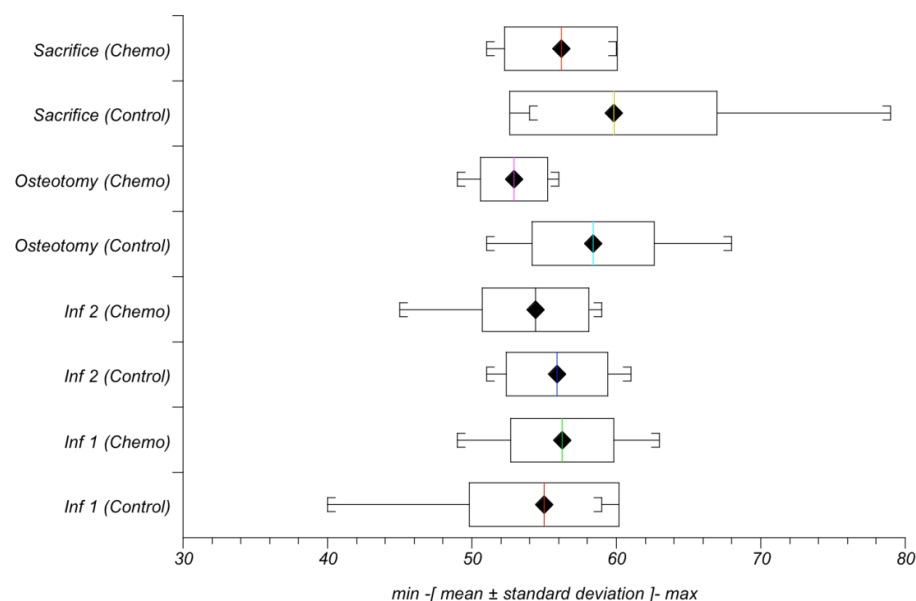
Power (for 5% significance) = 99.83%

Sacrifice

$p = 0.28$

95% confidence interval for difference between means = -3.25 to 10.51

Power (for 5% significance) = 38.91%



2.10.12 Adjuvant Group Total Protein (g/L)

		Mean	σ
Infusion 1	(Control)	55.55	1.92
	(Chemotherapy)	55.92	3.12
Osteotomy	(Control)	57.00	4.74
	(Chemotherapy)	55.25	3.91
Infusion 2	(Control)	55.00	0.71
	(Chemotherapy)	58.55	2.73
Sacrifice	(Control)	59.22	3.11
	(Chemotherapy)	58.27	3.80

Infusion 1

$p = 0.74$

95% confidence interval for difference between means = -2.64 to 1.90

Power (for 5% significance) = 7.3%

Osteotomy

$p = 0.35$

95% confidence interval for difference between means = -2.09 to 5.59

Power (for 5% significance) = 23.03%

Infusion 2

$p = <0.01$ (F test significant)

95% confidence interval for difference between means = -5.44 to -1.65

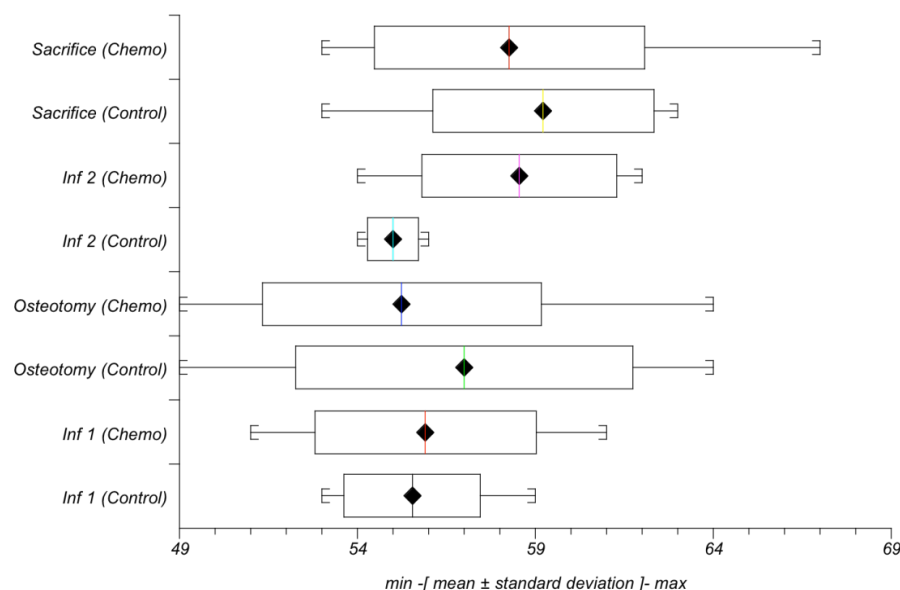
Power (for 5% significance) = 95.57%

Sacrifice

$p = 0.55$

95% confidence interval for difference between means = -2.36 to 4.26

Power (for 5% significance) = 11.87%



2.10.13 Neo-adjuvant Group ALT (U/L)

		Mean	σ
Infusion 1	(Control)	64.00	37.86
	(Chemotherapy)	41.09	12.10
Infusion 2	(Control)	80.80	40.37
	(Chemotherapy)	59.00	33.19
Osteotomy	(Control)	121.70	80.71
	(Chemotherapy)	44.91	9.62
Sacrifice	(Control)	102.00	65.25
	(Chemotherapy)	58.67	18.68

Infusion 1

p = 0.08 (F test significant)

95% confidence interval for difference between means = -2.09 to 47.91

Power (for 5% significance) = 39.71%

Infusion 2

p = 0.18

95% confidence interval for difference between means = -10.88 to 54.48

Power (for 5% significance) = 43.32%

Osteotomy

p = 0.02 (F test significant)

95% confidence interval for difference between means = 23.03 to 130.55

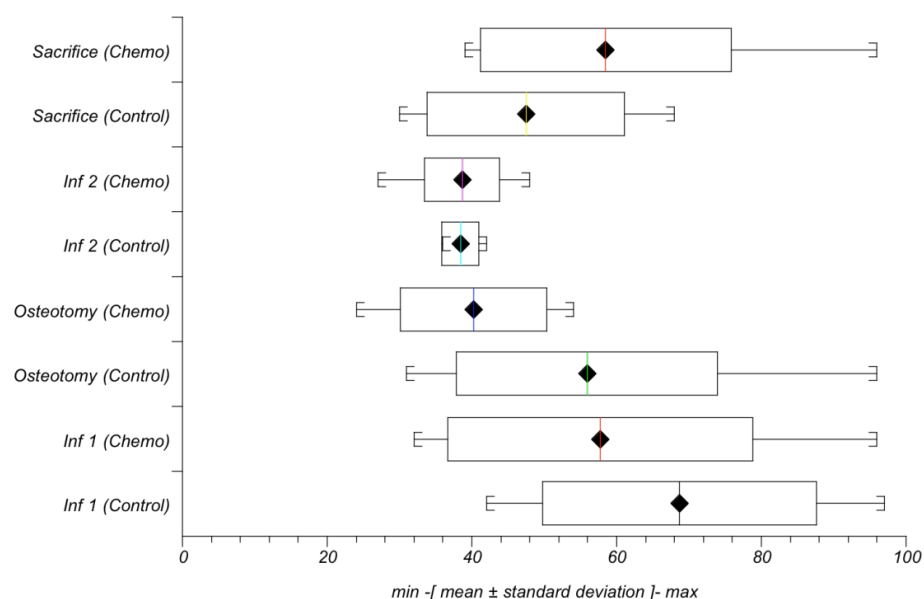
Power (for 5% significance) = 76%

Sacrifice

p = 0.14

95% confidence interval for difference between means = -13.48 to 100.15

Power (for 5% significance) = 25.97%



2.10.14 Adjuvant Group ALT (U/L)

		Mean	σ
Infusion 1	(Control)	68.64	18.93
	(Chemotherapy)	57.75	21.09
Osteotomy	(Control)	55.90	18.00
	(Chemotherapy)	40.27	10.11
Infusion 2	(Control)	38.40	2.51
	(Chemotherapy)	38.64	5.20
Sacrifice	(Control)	47.44	13.59
	(Chemotherapy)	58.50	17.33

Infusion 1

p = 0.21

95% confidence interval for difference between means = -6.55 to 28.33

Power (for 5% significance) = 40.15%

Osteotomy

p = 0.02

95% confidence interval for difference between means = 2.46 to 28.79

Power (for 5% significance) = 90.31%

Infusion 2

p = 0.93

95% confidence interval for difference between means = -5.55 to 5.08

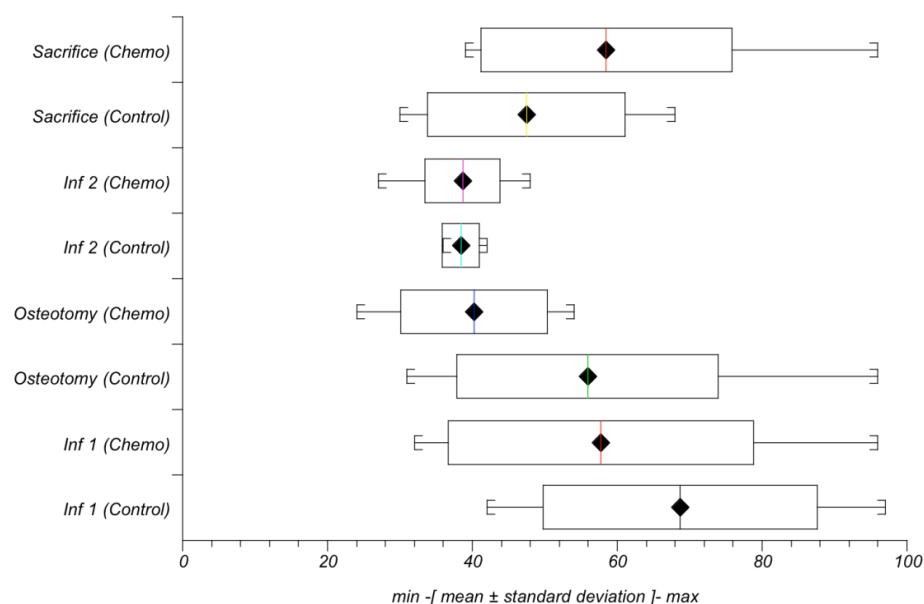
Power (for 5% significance) = 5.13%

Sacrifice

p = 0.14

95% confidence interval for difference between means = -26.26 to 4.15

Power (for 5% significance) = 51.25%



2.10.15 Neo-adjuvant Group γ GT (U/L)

		Mean	σ
Infusion 1	(Control)	8.25	4.39
	(Chemotherapy)	6.73	2.37
Infusion 2	(Control)	7.44	3.00
	(Chemotherapy)	6.08	4.32
Osteotomy	(Control)	11.30	6.34
	(Chemotherapy)	6.00	2.14
Sacrifice	(Control)	13.00	5.94
	(Chemotherapy)	10.29	2.56

Infusion 1

p = 0.32

95% confidence interval for difference between means = -1.58 to 4.63

Power (for 5% significance) = 28.72%

Infusion 2

p = 0.43

95% confidence interval for difference between means = -2.16 to 4.89

Power (for 5% significance) = 17.28%

Osteotomy

p = 0.03 (F test significant)

95% confidence interval for difference between means = 0.89 to 9.71

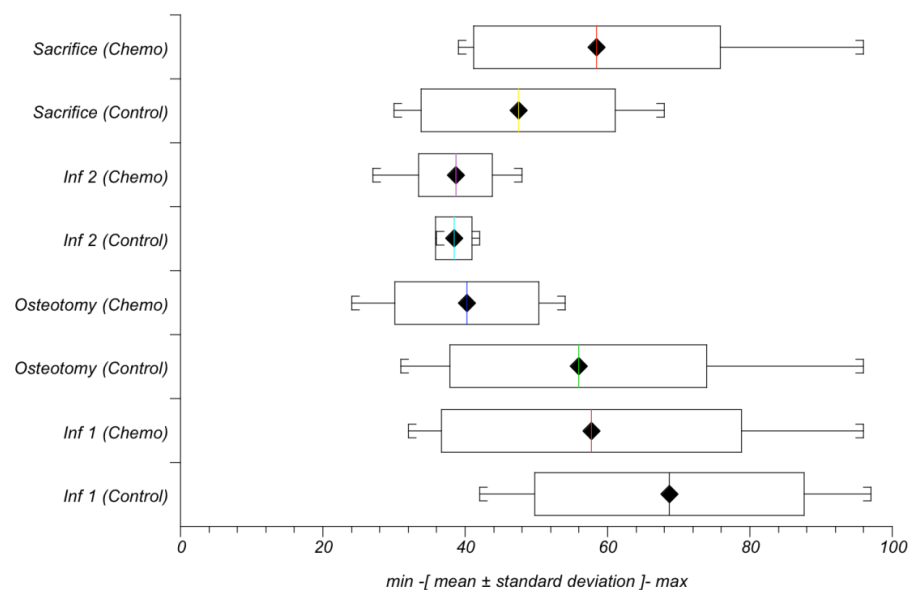
Power (for 5% significance) = 61.9%

Sacrifice

p = 0.29

95% confidence interval for difference between means = -2.62 to 8.05

Power (for 5% significance) = 29.71%



2.10.16 Adjuvant Group γ GT (U/L)

		Mean	σ
Infusion 1	(Control)	11.64	5.03
	(Chemotherapy)	10.67	3.60
Osteotomy	(Control)	9.20	2.86
	(Chemotherapy)	9.17	1.59
Infusion 2	(Control)	6.60	0.89
	(Chemotherapy)	8.27	1.10
Sacrifice	(Control)	6.11	1.76
	(Chemotherapy)	5.45	2.02

Infusion 1

$p = 0.60$

95% confidence interval for difference between means = -2.80 to 4.74

Power (for 5% significance) = 10.81%

Osteotomy

$p = 0.97$

95% confidence interval for difference between means = -1.98 to 2.04

Power (for 5% significance) = 5.02%

Infusion 2

$p = <0.01$

95% confidence interval for difference between means = -2.885285 to -0.460169

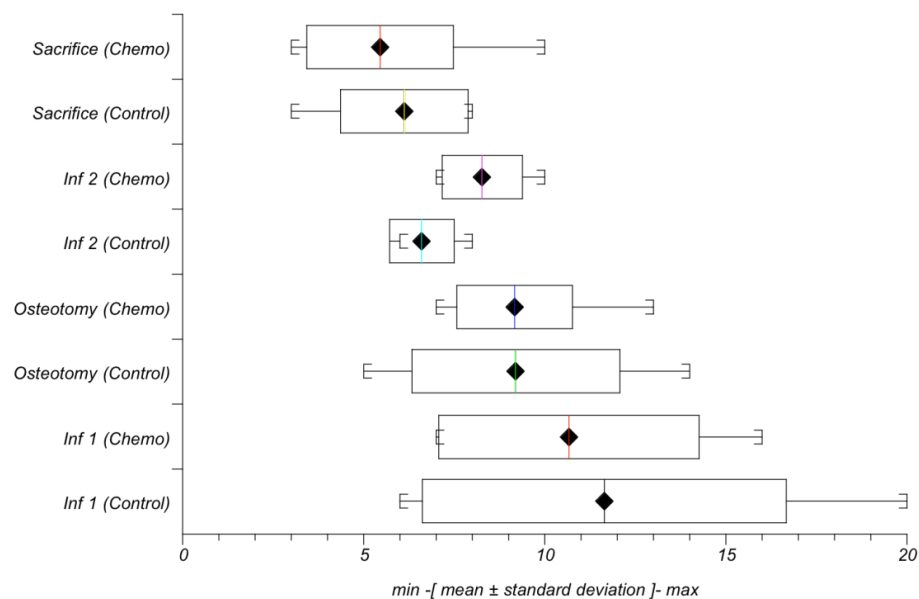
Power (for 5% significance) = 91.88%

Sacrifice

$p = 0.45$

95% confidence interval for difference between means = -1.15 to 2.46

Power (for 5% significance) = 16.29%



2.10.17 Neo-adjuvant Group Weight Gain (%)

		Mean	σ
Week 10	(Control)	24.07	10.07
	(Chemotherapy)	7.59	13.31
Week 12	(Control)	37.19	12.02
	(Chemotherapy)	18.88	7.27
Week 16	(Control)	54.32	8.65
	(Chemotherapy)	28.42	9.02

10 Weeks

$p = <0.01$

95% confidence interval for difference between means = -26.47 to -6.49

Power (for 5% significance) = 99.58%

12 Weeks

$p = <0.01$

95% confidence interval for difference between means = 9.90 to 26.72

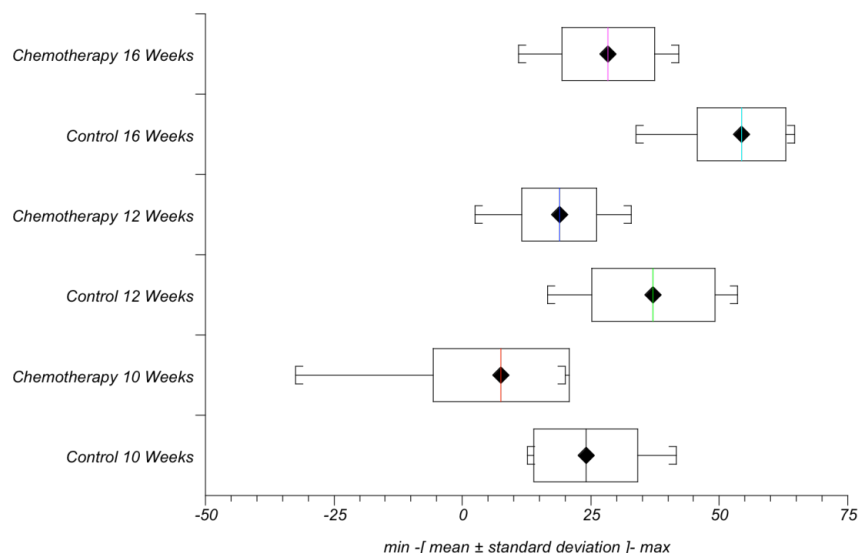
Power (for 5% significance) > 99.99%

16 Weeks

$p < 0.01$

95% confidence interval for difference between means = -33.56 to -18.23

Power (for 5% significance) > 99.99%



2.10.18 Adjuvant Group Weight Gain (%)

		Mean	σ
Week 12	(Control)	9.96	4.16
	(Chemotherapy)	5.22	6.56
Week 16	(Control)	16.16	5.58
	(Chemotherapy)	10.06	6.65

12 Weeks

$p = 0.03$

95% confidence interval for difference between means = 0.46 – 11.22

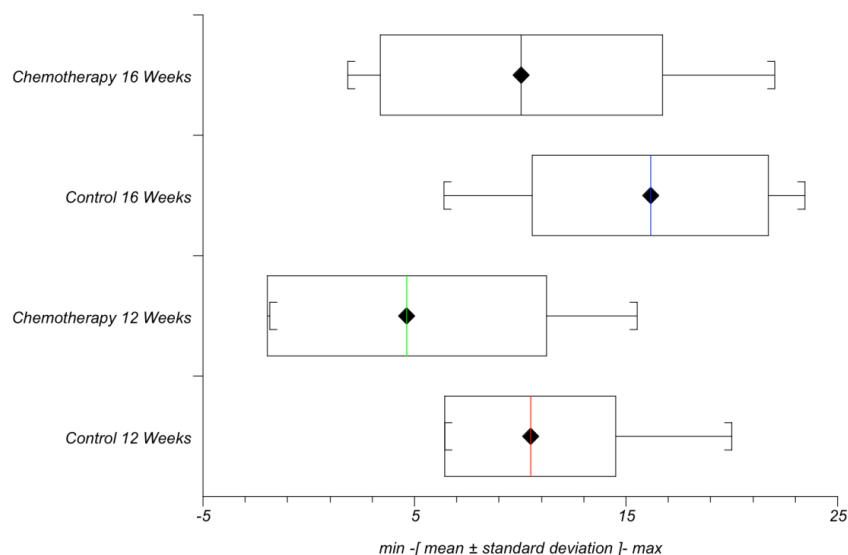
Power (for 5% significance) = 84.67%

16 Weeks

$p = 0.04$

95% confidence interval for difference between means = 0.24 – 11.95

Power (for 5% significance) = 79.93%



2.11 Overview of Dose Response Results

2.11.1 Haematological Function

The initial haemoglobin concentration was significantly lower in the control group of the neo-adjuvant arm. This trend had reversed by week 12 indicating a relative decrease in haemoglobin concentration following chemotherapy. This difference was no longer present at the conclusion of the experiment.

In the adjuvant arm, there was no statistically significant difference in haemoglobin concentration at the commencement of the experiment. There was a significant

reduction following both cytotoxic infusions, which had recovered at the conclusion of the experiment.

The relative anaemia was associated with a statistically significant reduction in haematocrit in the neo-adjuvant group, only at the time of osteotomy and was not seen in the adjuvant group at any point.

There were no statistically significant differences in total white cell count at any point in either arm of the experiment.

2.11.2 Renal Function

There was a statistically significant increase in serum urea concentration in the neo-adjuvant arm following the initial cytotoxic infusion, which had recovered at the conclusion of the experiment. There were no differences in mean creatinine concentration between the active and control groups in the neo-adjuvant arm.

There was a statistically significant increase in serum urea concentration in the cytotoxic group of the adjuvant arm at the commencement of the experiment, which persisted until the time of the osteotomy. There was a statistically significant increase in serum creatinine concentration in the adjuvant arm at the time of osteotomy and second infusion.

2.11.3 Hepatic Function

A statistically significant reduction in total protein was observed in the neo-adjuvant control group at the time of osteotomy and at the time of second infusion in the adjuvant chemotherapy group.

There was a statistically significant increase in ALT at the time of the osteotomy in control groups in both neo-adjuvant and adjuvant arms of the experiment.

There was a statistically significant increase in γ GT in the control group of the neo-adjuvant arm at the time of osteotomy with a reverse trend observed at the time of second infusion in the adjuvant group.

2.11.4 Weight Gain

There was a statistically significant decrease in percentage weight gained in the chemotherapy group of the neo-adjuvant arm of the experiment. A significant but less marked trend was observed in the adjuvant group.

2.11.5 Summary

The dose response experiment demonstrated that an initial infusion of 1mg / kg cis-platinum and 2mg / kg adriamycin and a second infusion of 1 mg / kg cis-platinum and 4 mg / kg adriamycin produced a statistically significant reduction in weight gain, irrespective of the timing of administration of the drugs. This combination did not produce biochemical evidence of renal or hepatic failure and surgery was possible without clinical evidence of infection.

This schedule proved to be robust and satisfied the primary requirements of the experimental model

1. Reproducible anaesthesia
2. Reproducible infusion of cis-platinum and adriamycin
3. Appropriate cytotoxic effect
4. Normal renal function
5. Normal hepatic function

The model was used to investigate the structural and material properties of regenerate bone and the experimental considerations and results are presented in the next section.

Section 3 The Effect of Cytotoxic Drugs on Regenerate Bone

The key surgical events in the management of osteogenic sarcoma are tumour excision and limb reconstruction. For the purpose of the animal model, osteotomy and distraction was used as a surrogate for surgical reconstruction and the effect and timing of cytotoxic drugs formed the subject of this component of the thesis. Two conditions were investigated with administration of 2 cycles of cis-platinum and adriamycin before osteotomy and lengthening or immediately before osteotomy and during the distraction period.

This created experimental conditions that simulated neo-adjuvant and adjuvant prescriptions. The models were simplistic in terms of the components and total number of cycles but provided a useful tool to perform a preliminary investigation into this problem.

3.1 Experimental Considerations

The osteotomy was performed aged 12-weeks in both groups and the interval between chemotherapy infusions was constant at 2-weeks. The timing of infusion varied between groups to simulate a neo-adjuvant and adjuvant strategy. The experimental conditions were identical in the control group for each arm, with an identical volume of N saline infused under identical conditions.

General health was assessed and markers of metabolic function were measured throughout the experiment. The lengthened tibiae were evaluated with plain radiographs, DXA and single cycle compression testing to failure.

3.1.1 Application of External Fixator and Osteotomy

Kaweblum *et al* (Kaweblum et al., 1994) investigated the timing of growth plate closure in juvenile NZW rabbits and demonstrated histological evidence of distal femoral physeal arrest at 20–23-weeks of age. Animals in both arms of the study were sacrificed at 16-weeks of age, representing approximately 75% completion of growth.

The right leg was shaved and prepared with Chlorhexadine 0.5%/Alcohol 70% (Delta West Pyt. Ltd., Bentley, WA Australia) and the operative field was occluded

with sterile surgical towels. A medial longitudinal skin incision was used with subperiosteal exposure of the tibia along its length.

Orthofix (Orthofix, Bussolengo, Italy) 3 mm external fixator pins were inserted 1.5 cm proximal and distal to the mid tibial diaphysis. A standard jig and power drill draped in a sterile container was used and the positions of the remaining 2 pins were determined by the jig. The configuration of the external fixator required the paired pins to be accurately positioned along the length of the tibia to avoid rotational mal-alignment, which would cause cantilever bending when the pins were clamped. The juvenile NZW Rabbit tibia is brittle and a mid-diaphyseal osteotomy was performed initially with multiple drill holes and was completed with bone cutters. (Figure iv)

An M100 mono-lateral fixator (Orthofix, Bussolengo, Italy) was attached approximately 1.5 cm from the skin, to accommodate for post-operative swelling and the wound was closed with interrupted sutures. (Figure v)

The wound was dressed with Povidone Iodine ointment (Professional Disposables Inc. Orangeburg, New York) and the animal was observed until alert and drinking before being returned to its cage.

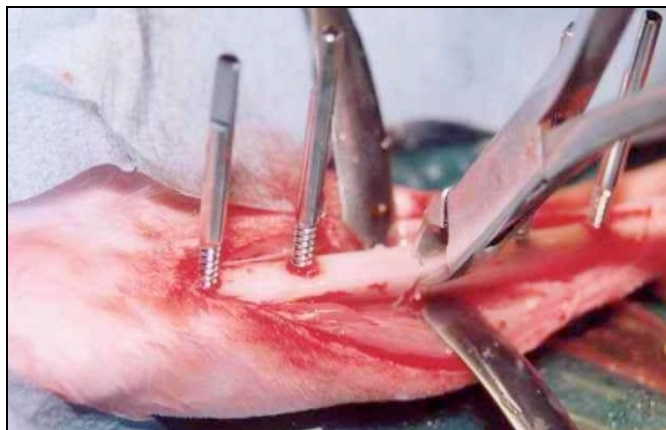


Figure iv Tibial osteotomy and placement of paired parallel 3mm pins

3.1.2 Distraction Phase

A latent period of 5-days is standard practice in children and adolescents undergoing limb reconstruction by distraction histiogenesis. The bone healing time

in a juvenile rabbit however is significantly shorter, with consolidation of regenerate within 4-weeks. This indicates rapid bone metabolism in this age group with a risk of premature consolidation if an equivalent latent period were used in a rabbit model. A latent period of 24-hours was chosen to imitate the human situation and although previous studies (Kojimoto et al., 1988) have suggested that this may cause a retardation of callus formation, this complication was not encountered.

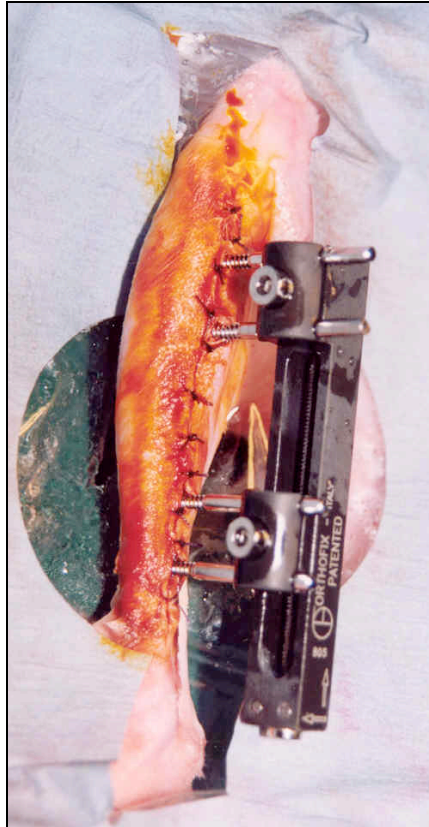


Figure v Orthofix M 100 External fixator in situ following mid diaphyseal tibial osteotomy

The fixator was distracted by one half turn every 12-hours which resulted in an incremental lengthening of 0.75 mm/day. This continued for 10-days, producing an overall lengthening of 7.5 mm. (Figure vi)

Consolidation of the regenerate occurred over a period of 18-days following distraction, at this point all animals were sacrificed with an intravenous injection of pentobarbitone sodium (120 mg / kg) (Valabarb Jurox Pty. Ltd., Silver Water, NSW, Australia).



Figure vi Incremental lengthening of 0.375 mm/12 hours after a 24-hour latent period.

3.2 Outcome Measures

3.2.1 Plain Radiography

The right hind limb was disarticulated and transported fresh to the Radiology department at the New Children's Hospital (Westmead NSW). The limb was aligned by an experienced radiographer (LC) to produce consistent cranio-caudal and medio-lateral images with a Siemens Multix H/UPH configuration using a Siemens digital luminous radiography cassette (18 x 24 cm) with a focus to film distance of 100 cm and a 50 kV (+/- 2mV) and 4 mA exposure. Hard copy images were produced with a Dry-View DVP Laser printer (Figure vii).

The alignment in the antero-posterior and lateral planes was measured with a goniometer and the oblique plane deformity was calculated using the graphical method described by Paley *et al.* (Paley, 2002)



Figure vii A) Lateral and B) Antero-posterior radiographs following lengthening

3.2.2 Dual Energy X-Ray Absorptiometry

DXA is a non-invasive method used to measure the mineral content of bone. A collimated beam of photons generated by an X-ray tube passes through the bone. This beam contains two discrete energies, distinguishable on the basis of their attenuation by bone and soft tissue. One serves as a reference for soft tissue thickness, the second is predominately attenuated by bone and is used to measure the mineral content expressed as bone mineral content (BMC) and areal bone mineral density (BMD). Volumetric bone mineral density (ν BMD) is derived from the BMD and the dimensions of the regenerate.

DXA has been used widely to investigate the composition of small animal bones, including rodents (Mitlak et al., 1991) and dogs. (Markel and Chao, 1993, Markel et al., 1991) DXA has been used to evaluate the material properties of bone formed in a rabbit model of distraction of the mandible (Persing et al., 1991) and tibia, (Hamanishi et al., 1995) the effect of rhBMP-2 on bone formation in a rabbit femoral defect (Laffargue et al., 1999) and the contribution of periosteum and bone marrow to bone lengthening. (Guichet et al., 1998)

An *et al* (An et al., 1994) investigated the relationship between the compressive strength of tricortical iliac crest grafts and BMD of the iliac crest and pelvis in humans using DXA. There was a high correlation between the ultimate load to

failure and compressive stress of the graft to the BMD of the intact pelvis and each graft. This suggested that the mechanical properties of bone are related to BMD, potentially providing a non-destructive method of assessment in clinical practice.

Eyres *et al* (Eyres et al., 1993b) used DXA to measure BMC and BMD to evaluate the extent and rate of new bone formation over an 18-month period, before, during and after lengthening 10 leg segments in 6 patients. They identified a short delay in consolidation after maximal distraction with accelerated mineralisation in most cases over the initial 3-months, followed by slower bone formation in later stages of lengthening.

This group (Eyres et al., 1993a) subsequently used DXA, ultrasonography and plain radiography to study the rate of formation of bone during lengthening of 17 segments in 10 patients. New bone was identified by DXA and ultrasonography one to 2-weeks after osteotomy but not until 4 to 8-weeks by plain radiography. Limb alignment and distraction could be measured by DXA throughout the period of lengthening. Ultrasonography demonstrated the distraction gap as an echolucent window, with new bone appearing as echogenic islands, which became aligned longitudinally and progressively filled the window. Ultrasonography could only measure the gap difference during the early stages of distraction when the edges of the corticotomy were still well defined but was useful in defining cystic defects in the regenerate which were not recognised by DXA or plain radiography.

Maffulli *et al* (Maffulli, 1997) studied the rate of regenerate BMC using DXA in callotasis lengthening of the lower limb in 11 children. From analysis of time graphs, a direct correlation emerged between early bone formation and subsequent bone mineral content accretion and 3 patient groups (slow, normal and fast) were identified. The pattern of accretion was not related to the fixator used, time or bone lengthened and appeared to be dependent on the underlying pathology. This suggests that the rate of mineral accretion is defined early in the lengthening process and administration of cytotoxic agents around the time of osteotomy may alter subsequent bone formation.

Reichel *et al* (Reichel et al., 1998) compared DXA and mechanical parameters in an ovine model of distraction osteogenesis using non-destructive axial compression testing and a torsional force to failure. They did not demonstrate a significant correlation between axial compression and BMD, however there was a strong correlation between maximum torque and BMD.

Chotel *et al* (Chotel et al., 2008) compared DXA measurements and 3-point bending assessment of bone stiffness in children undergoing lengthening by distraction osteogenesis. There was a poor correlation for absolute DXA data but a linear correlation was identified between BMC measurements and stiffness, if BMC was expressed as a percentage of a symmetrical region of the contralateral side

The literature discussed in previous paragraphs suggests that DXA is a valid method of evaluation of the material properties of the regenerate bone. It was readily available and inexpensive and provided a practical method of comparison between control and treated animals.

3.2.2.1 DXA Measurements

Bone density measurements were made by an experienced radiographer (JB) using a total body dual-energy X-ray densitometer (LUNAR DPX, LUNAR Radiation Crp., Madison, WI) with software designed for measuring small animals (LUNAR DPX, Small Animal Software version 1.0, LUNAR Radiation Crp., Madison, WI). This machine used a constant potential X-ray source (76 kV) and a K-edge filter (cerium) to produce stable dual-energy X-rays with effective energies of 38 and 70 keV.

The disarticulated right hind limb was assessed with soft tissues intact and the scan length was determined by the length of the bone (the scan area was approximately 50 mm x 130 mm). Each leg was placed in a supine position on the scan table and an anterior-posterior scan was performed. The leg was then medially rotated through 90 degrees and a lateral scan was performed. BMD, BMC and bone area (BA) values were obtained with the “Manual Analysis” facility. The height of the boxes was determined by the length of the regenerate and all box widths were identical (15 mm).

The distance from the knee to the top of the regenerate was measured with a software ruler, and the length of the regenerate were measured and recorded. A “Region of Interest” (ROI) box was placed over the regenerate. The height of the ROI was such that the entire length of the regenerate was included. The boxes were positioned so that all the bone and some soft tissue were included in the region of interest. (Figure viii)



Figure viii DEXA Image with corresponding radiograph

The software calculated BMC (g), BMD (g/cm²), average bone width (cm) and average bone area (cm²). vBMD was calculated assuming that the bone is an elliptical cylinder.

The area of the cross-section of bone =

$$\pi \times \frac{(\text{AP Bone Width})}{2} \times \frac{(\text{Lat Bone Width})}{2}$$

The volume of the region V = Cross-sectional Bone Area x ROI Height

$$\text{Volumetric BMD (g/cm}^3\text{)} = \frac{\text{BMC}}{V}$$

Each scan produced the following data from the AP and Lat scans

Bone Width (cm)	(W)
Bone Area (cm ²)	(A)
Bone Mineral Content (g)	(BMC)
Bone Mineral Density (g/cm ²)	(BMD)
Volumetric Bone Mineral Density (g/m ³)	(vBMD)

From these data the following were calculated

Mean BMC (g)	$\frac{(\text{Lat BMC} + \text{AP BMC})}{2}$
Mean BMD (g/cm ²)	$\frac{(\text{Lat BMD} + \text{AP BMD})}{2}$
Mean vBMD (g/cm ³)	$\frac{(\text{Lat vBMD} + \text{AP vBMD})}{2}$

3.2.3 Mechanical Testing

The mechanical testing of bone, whether intact or during fracture healing has been a subject of investigation for over a century. Wertheim (Wertheim, 1847) determined the maximum load in tension for small prepared specimens of human bone in 1847, whilst other investigators (Hulsen, 1896, Rauber, 1876) used static tensile and compressive tests but did not specify the origin of the bone and the experimental procedure used.

In the middle of the 20th century, the interest in bone characterisation increased with particular emphasis on the measurement of the anisotropy of mechanical properties. (Evans and Lebow, 1951, Evans and Lebow, 1952)

Sedlin *et al* (Sedlin and Hirsch, 1966) highlighted the effects of specimen preservation, temperature, geometry and age of donor on the mechanical properties of bone and Kraus (Kraus, 1968) documented the mechanical properties of machined specimens of excised cortical bone. The field of whole bone testing is less well documented and experimental values are more difficult to analyse, usually representing an average of an anisotropic property.

Burstein *et al* (Burstein and Frankel, 1971) described 5 criteria which they considered to be pre-requisites for a standard test of intact bones in experimental animals.

- 1) The loading configuration should produce fractures similar to clinical fractures.
- 2) The loading configuration should subject the bone to equally severe loading conditions at every section along its length, to be able to identify weak sections.
- 3) The loading mode must not be critically dependent upon bone geometry, in particular bone length, in terms of the severity of its effect.
- 4) The loading configuration must allow control of the rate of application of load, so that the time conditions produced in the test will be reproducible and preferably representative of those of normal trauma.
- 5) The loading configuration should result in a test apparatus that is relatively inexpensive and may be operated by persons with only ordinary skills in handling such mechanical equipment.

They considered that axial compression and tension did not meet criteria (1) and (5) and stated that whilst long bones were loaded in axial compression, they seldom failed because of this loading. This paper is often cited in clinical and experimental practice but it describes idealised conditions that are difficult to define and replicate in the clinical context.

Previous studies have evaluated the biomechanics of intact bone, lengthened bone, excised bone segments and implanted bone. These have utilised a spectrum of testing configurations including tension, compression, torsion and 3-point bending. There is no consensus of experimental design and the justification for a particular method of testing is seldom discussed.

3.2.3.1 Tensile Testing

Black *et al* (Black *et al.*, 1984) evaluated the healing characteristics of a rabbit fibular osteotomy. Excised specimens were harvested at intervals and loaded in tension until failure. This demonstrated a rapid return of stiffness, which occurred within 16-days of osteotomy and correlated with the radiological appearance of the callus. This suggested that tensile testing minimised artefact and permitted direct

intrinsic determinations of tissue quality. The authors recognised that this method of testing was associated with severe limitations and in particular, there were significant technical difficulties associated with the bone-clamp interface.

Walsh *et al* (Walsh et al., 1994) investigated the biomechanical properties of lengthened bone in a canine model of tibial lengthening. The tibiae were embedded in polymethylmethacrylate (PMMA) in aluminium moulds and the tensile mechanical properties were determined using a Model 810 Materials Testing Machine. Lengthened and control bones were tested at a rate of 50% working length/minute. The peak load to failure, energy to peak load and stiffness were measured at 3, 6, 9 and 12-weeks. Linear regression analysis demonstrated a strong correlation between peak load and stiffness with time after completion of lengthening.

3.2.3.2 Torsional Testing

Collier *et al* (Collier et al., 1976) used destructive torsional testing to determine whether porous-coated Vitallium intramedullary rods could be used to bridge segmental defects in a rabbit tibia. The model used a silastic spacer to bridge a 1 cm defect in the mid shaft of the tibia with a rod inserted through the proximal tibial segment, through the spacer and into the distal bone segment. The animals were sacrificed 30-weeks postoperatively and the mechanical properties of the tibia from each rabbit were measured in a torsional testing machine. The load at failure of the tibia with the segmental defect averaged 90% of the contralateral control tibia, suggesting that bone ingrowth occurred under dynamic loading. The authors concluded that porous coated intra-medullary rods might provide a method of reconstruction of segmental bone loss.

Paavolainen *et al* (Paavolainen, 1978) used torsional testing to demonstrate that the rabbit tibio-fibular complex exhibited right to left differences in energy absorption capacity, torsional rigidity, torque moment at fracture and angular deformation. In response to this observation and to remove asymmetry as a confounding variable, the right tibia was lengthened in the experimental component of this thesis.

Burchardt *et al* (Burchardt et al., 1983) used destructive torsional testing to evaluate the effect of adriamycin and methotrexate on the mechanical properties of fibular autograft reconstruction in a canine model. Thirty-two male mongrel dogs were used to compare the effect of adriamycin, methotrexate and an untreated group with a contralateral sham-operated limb.

All grafts and sham segments were harvested at 6-months and tested with a rapid-loading torsion machine to determine load to failure. This was analysed using actual values and the percentage difference between the graft and the sham segments of each dog in each group.

Higher doses of adriamycin resulted in grafts that were mechanically weaker than normal. There was a reduction in the torque to failure, which was due to a combination of new bone suppression and peripheral graft reabsorption. The authors concluded that inhibition of new bone formation may prolong the interval required for osseous graft-host union, and there may be a greater incidence of graft-host non-union.

Pilla *et al* (Pilla et al., 1990) investigated the effect of ultrasound on the healing of a NZW rabbit fibular osteotomy. All bones were subjected to destructive torsional testing to produce a torque verses angular displacement curve. The maximum torque was represented by the peak of the curve immediately before failure. This study demonstrated that non-invasively applied ultrasound for 20-minutes daily, accelerated healing by a factor of approximately 1.7. The authors discussed the limitations of torsional analysis and in particular, the effect of increased surface area of the callus in osteotomised animals on the stiffness of the osteotomised specimens, which was greater than intact bone.

Fyda *et al* (Fyda et al., 1995) used a rabbit femur to study the time-dependent mechanical and radiographic changes with various treatments of surgically created windows. Specimens were loaded to failure on a torsional testing apparatus at a constant rate of 30°/second to obtain values for ultimate torque, maximum angle of deformation, and energy capacity expressed as percent of paired control. The experiment demonstrated that replacement of a cortical window resulted in significantly greater whole bone strength, which was time dependent.

3.2.3.3 Bending Tests

Bending tests (3-point, 4-point, or cantilever) are frequently used to assess the mechanical strength of bone as they are relatively straightforward and allow rapid analysis. Arguments in their favour usually emphasise that bending is the most common mode of deformation *in vivo*. This technique involves complex internal stress fields and produces results that are strongly dependent on testing skill and orientation of the test specimen. Furthermore, when applied to fractures or experimental osteotomies, there is a tendency to combine the properties of the bone with those of the developing callus in an unpredictable manner.

Terjesen *et al* (Terjesen and Benum, 1983) used 3-point bending within the elastic range to study the *in vitro* stress-protecting effect of an external fixator on osteotomised rabbit tibiae. An Instron Testing machine (Type 1123) was used, with a constant rate of deformation of 5 mm/minute applied to the anterior edge of the bone. The osteotomy was performed with an oscillating saw at a standard anatomical site, allowing for precise positioning of the applied load. The BMC was measured by photon absorptiometry.

The strength and stiffness were reduced to 87 and 88% of controls after 12-weeks. The mineral content in the bone segment, which had been stabilised by external fixation, was significantly reduced after 12-weeks. There was no significant change in BMC distal to the external fixation device.

Hanafusa *et al* (Hanafusa et al., 1995) investigated the mechanical strength, bone mineral content and density in rabbit femoral osteotomies stabilised with either ultra high strength poly-L-lactic acid or stainless steel plates. The mechanical strengths of the paired femora were measured by 3-point bending using a Shimadzu Autograph SD-100 with load applied at the site of a transverse midshaft osteotomy. In the poly-L-lactic acid group, the mechanical strength of the specimen was identical to the untreated femur by 25-weeks. The stainless steel group showed significantly lower mechanical strength with osteopenia due to stress shielding after 25-weeks.

An *et al* (An et al., 1996) investigated mechanical symmetry in NZW rabbit long bones with 3-point bending for diaphyseal bone and indentation for epiphyseal bone. The loading point was defined as the mid shaft of the femur, junction of

middle and distal thirds of the tibia and the junction of the proximal $\frac{3}{5}$ and distal $\frac{2}{5}$ the humerus. This demonstrated that the mean mechanical values for long bones were symmetrical and produced a normal comparable database for bending and indentation parameters of the rabbit femur, tibia, and humerus.

Ayres *et al* (Ayers et al., 1996) investigated the relationship between the physical, structural and material properties of femora and tibiae of mice, rats, rabbits and cats. Physical parameters included total bone length, mid-diaphyseal cortical area, body mass and bone dry mass. Three-point bending was performed with an Instron 1331 testing servo hydraulic testing system with a deflection rate of between 1 and 5 mm/minute depending on the species. Adjustable supports were positioned in a constant configuration at the proximal and distal diaphysis to measure structural (stiffness, elastic strength, maximum strength) and material properties (modulus of elasticity, elastic stress and elastic strain). Linear regression analysis was used to correlate the physical and mechanical properties and demonstrated that dry mass was a significant predictor of the structural properties of bone from these species.

3.2.3.4 Compression Testing

Ohyama *et al* (Ohyama et al., 1994) investigated the mechanical behaviour of regenerate in a Japanese White Rabbit model of femoral lengthening. Loading and unloading hysteresis and stress relaxation tests were used to investigate regenerate produced after continuous and discontinuous distraction. This demonstrated a time dependant effect, with viscoelastic behaviour in callus formed by continuous distraction and elastic behaviour in the discontinuous group, corresponding to the rest period. The authors correlated the mechanical behaviour with histological examination of retrieved specimens. In the continual distraction group, maintenance of viscoelastic properties was associated with central undifferentiated connective tissue. In the discontinuous group, the elastic properties were associated with central replacement of connective tissue with cartilage and bone.

An *et al* (An et al., 1994) investigated the correlation between the mechanical and structural properties of tri-cortical bone grafts under compressive load and demonstrated a linear relationship between load to failure and compressive

strength. The potential clinical application of this relationship was the use of DXA to predict the strength of iliac bone grafts in anterior interbody spinal fusion.

3.2.3.5 Overview of Available Methods of Mechanical Testing

The literature describing the optimum method of mechanical evaluation of bone is ambiguous. Tensile testing tests (Black et al., 1984, Walsh et al., 1994) depend on the original geometry of the bone, which is highly variable, even within a single species and age group. Analysis is also influenced by the integrity of the bone-clamp interface and is a difficult technique to reproduce.

Bending tests (An et al., 1996, Ayers et al., 1996, Hanafusa et al., 1995, Terjesen and Benum, 1983) involve complex internal stress fields and produce results that are influenced by the testing skill and orientation of the specimen. Bending tests also present technical difficulties associated with sample positioning which in the presence of non-uniform regenerate, would lead to unacceptable testing errors.

Torsional testing has been used in previous studies (Collier et al., 1976, Fyda et al., 1995, Paavolainen, 1978, Pilla et al., 1990, Burchardt et al., 1983) and is a useful method of analysis for intact bone but was not appropriate for analysis of regenerate bone due to the mode of failure.

Failure occurs on the compression side of an eccentrically loaded bone, leading to progressive bending of regenerate, which is observed in clinical practice. This mode of failure is simulated by axial compression, which was considered most representative of the *in vivo* mode of failure. Previous studies (An et al., 1994, Ohyama et al., 1994) have demonstrated a relationship between structural and mechanical properties of bone with this type of compression testing and a simple uni-axial loading test provided a reproducible method of analysis for this experiment.

3.2.4 Mechanical Testing Used in this Experiment

The logistical constraints of the experiment necessitated freezing the excised tibiae after DXA analysis. Roe *et al* (Roe et al., 1988) demonstrated that freezing sterile canine cortical allografts at 20°C for 32-weeks did not alter the compressive load to failure. Griffon *et al* (Griffon et al., 1995) however, demonstrated that

energy absorbed at failure and ultimate displacement in canine metacarpals, metatarsals and ribs frozen in isotonic saline solution were increased by 25 – 30% and 18 – 24% respectively compared to bones that were dry frozen. Cortico-cancellous grafts frozen in normal saline solution were less fragile and brittle than grafts stored in plastic without saline solution.

To eliminate the potentially confounding effect of freezing on the mechanical properties of bone, all the harvested tibiae were processed in an identical manner. The soft tissues were excised from each of the lengthened (right) tibiae, the specimens were stored in saline and frozen at -20°C and thawed at room temperature for 6-hours before testing.

Mechanical tests were performed in the Department of Mechanical and Mechatronic Engineering, University of Sydney by Mr J Wong Lee (JWL), Undergraduate Engineering Student in partial fulfilment of the requirements for the Degree of Bachelor of Engineering (University of Sydney 1996), supervised at all stages by FM. The experiment required reproducible longitudinal alignment of the bone during compression testing and this was achieved using a mounting block designed by JWL under the supervision of FM. The mounting block consisted of two cylinders with an adjustable connector to accommodate variable bone lengths (Figure ix, Appendix 2).

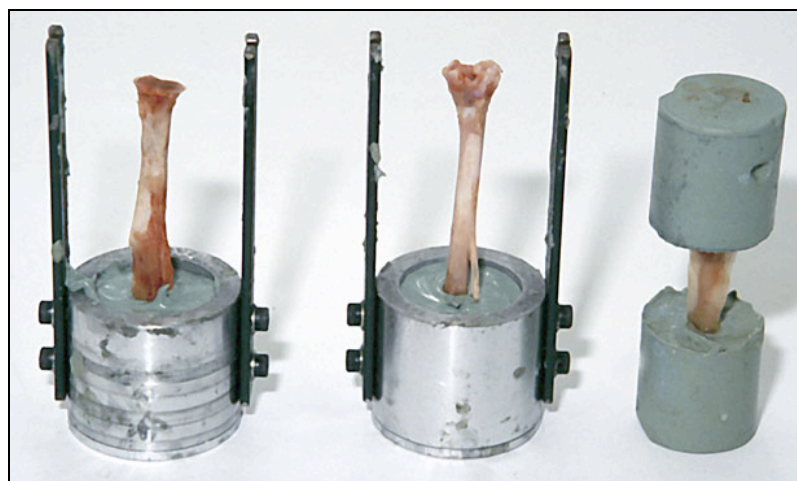


Figure ix Tibial Mounting Block

The tibiae were mounted in Permatex Autobody Filler (Permatex Inc, Hertford, Connecticut USA). Permatex is a 2 compound polyester resin, which was

analysed by compression testing, prior to its use in this experiment (Lee, 1996). Permatex resin was loaded in compression at 2 mm/min until failure and could be loaded to 5 kN without exceeding the elastic limit. The maximum load to failure for the rabbit tibia in all groups was 1.85 kN (Rabbit T) demonstrating that test bones failed at substantially lower loads than the polyester resin.

3.2.4.1 Rate of Loading

The material characteristics of mammalian bone are influenced by the loading environment and this has implications on the method of testing.

McElhaney *et al* (McElhaney and Byars, 1965) investigated the response of cubes of bovine bone to compression loads at various strain rates and found that Young's modulus increased, ultimate stress increased, and strain to failure decreased with increasing strain rate. Wright *et al* (Wright and Hayes, 1976) demonstrated different mechanisms of bone failure that were dependent on the rate of applied load. At slow strain rates (5.0×10^{-4} /sec) bone initially displays elastic deformation, followed by non-elastic deformation prior to failure. At higher strain rates (5.0×10^1 /sec) bone exhibited elastic properties until the point of failure.

Rubin *et al* (Rubin and Lanyon, 1982) concluded that physiological strain rates during walking and running for horses and dogs was in the range of 0.005–0.08 s^{-1} and that this was also a realistic range also for humans. Lanyon *et al* (Lanyon *et al.*, 1975) measured strain rates of 0.013 s^{-1} in humans during running and Burr *et al* (Burr *et al.*, 1996) measured maximum strain rates of 0.050 s^{-1} during sprinting and downhill running.

The experiment aimed to recreate loading characteristics of normal walking and a rate of loading of 2 mm/min was selected to reproduce a slow strain rate consistent with previous studies. This was validated by the consistent pattern of failure, which involved an initial elastic phase and non-elastic deformation prior to failure. (Figure xii)

3.2.4.2 Preparation and Mounting

The ankle and knee were disarticulated and all soft tissues were removed, leaving the tibio-fibular complex. This was embedded in the mounting block described in section 3.2.3.1, placed in an Instron mechanical testing machine (Instron Corporation, Massachusetts USA) and loaded in compression to failure at a rate of 2 mm/min using a 10 kN load cell (Figure x). The gauge length was determined by the position of the screw holes adjacent to the regenerate bone and measured approximately 3.5 – 4.0 cm. This configuration was chosen to standardise the mounting and to support the screw holes, which may have acted as a stress riser. Displacement (mm) for increasing load (Newtons) was measured until failure and data were saved in an Excel spreadsheet and exported to Easy Plot (Spiral Software Norwich, Vermont USA) and load displacement curves for each specimen were produced (Figure xi).

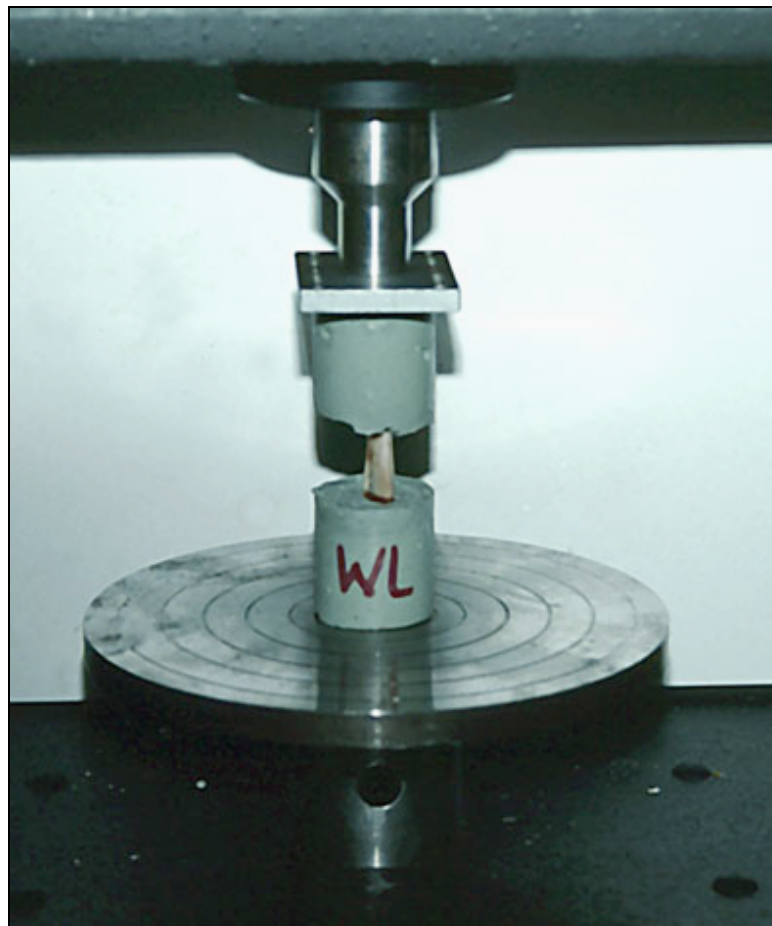


Figure x Compression Testing

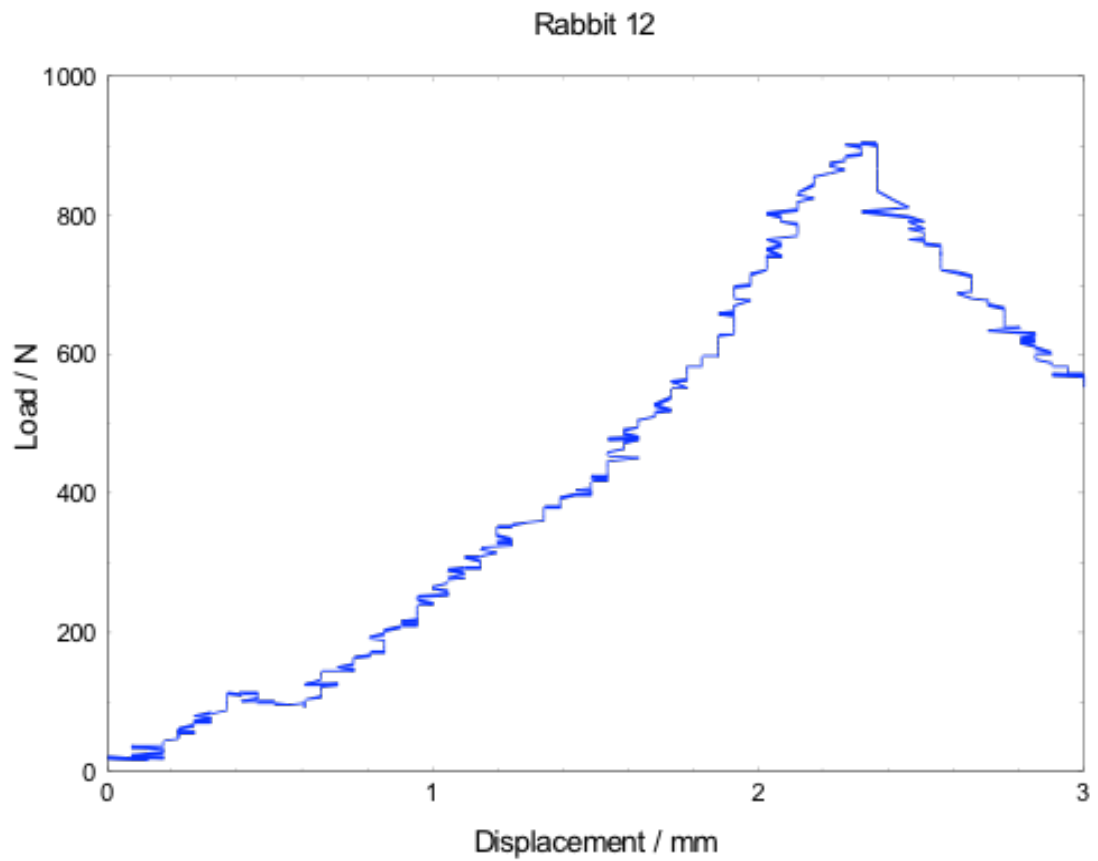
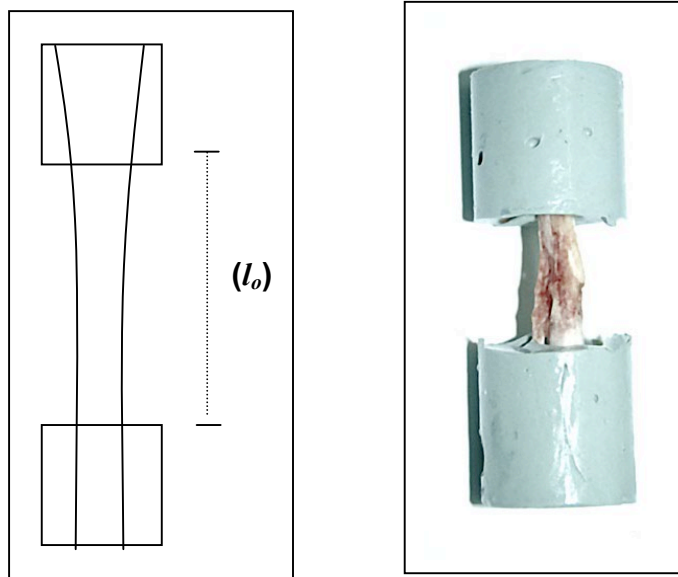


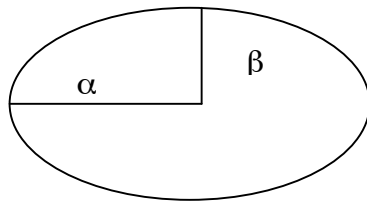
Figure xi Load / Deformation Plot

3.2.4.3 Stress/Strain Analysis

The length of exposed bone (l_o) was measured before compression testing. This was standardised during mounting, ensuring that the polyester resin covered the screw holes adjacent to the regenerate.



It was assumed that the regenerate was elliptical and the mean area was calculated from DXA measurements in the antero-posterior and medio-lateral planes using the following formula;



$$Area = \pi\alpha\beta$$

The load/displacement data was transformed to produce stress/strain data using the following formula;

$$\frac{\text{Stress}}{\text{Strain}} = \frac{\text{Load/Area}}{\text{Displacement/} l_0}$$

The data were transferred to Easy Plot (Spiral Software) and Stress-Strain curves were produced for each specimen. (Figure xii)

The Modulus of Elasticity is defined by the formula;

$$\frac{(\sigma_1 - \sigma_2)}{(\epsilon_1 - \epsilon_2)}$$

Where σ_n and ϵ_n are defined as equivalent co-ordinates within the elastic region, and illustrated in figure xii (---).

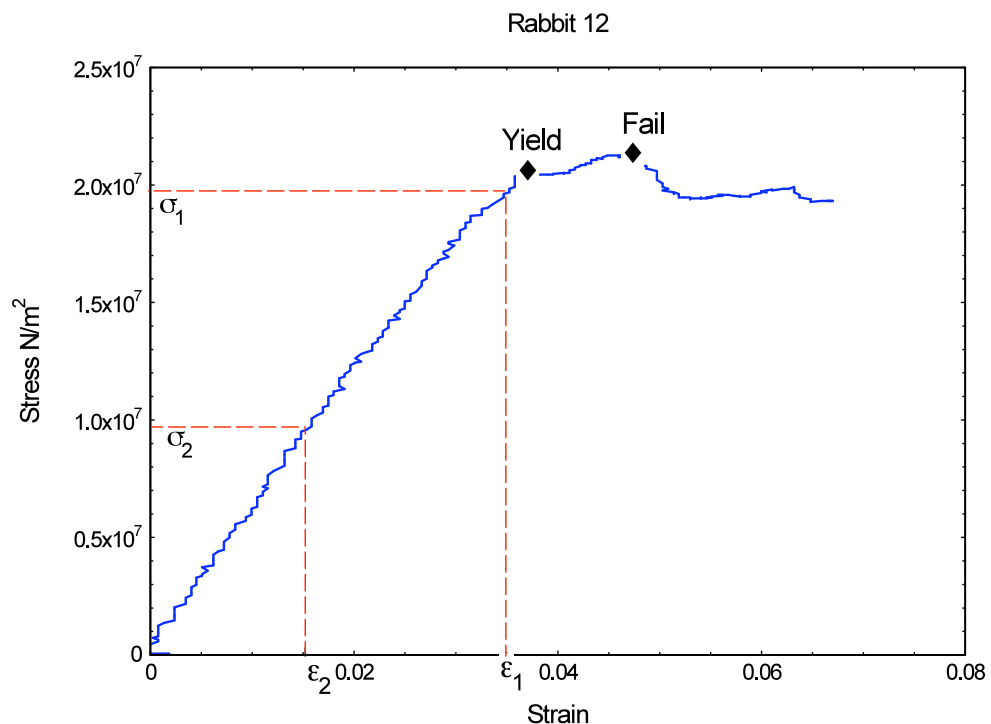


Figure xii

Stress / Strain Plot

The energy at yield and failure were represented as the area under the curve at each point and were calculated by integration.

In some cases, there was an initial increase in strain without an associated change in load and it was assumed that this represented bedding down between the bone and jig. (Figure xiii) In this situation, the origin was recalibrated and the linear area was isolated using the data capture facility of the Easy Plot software.

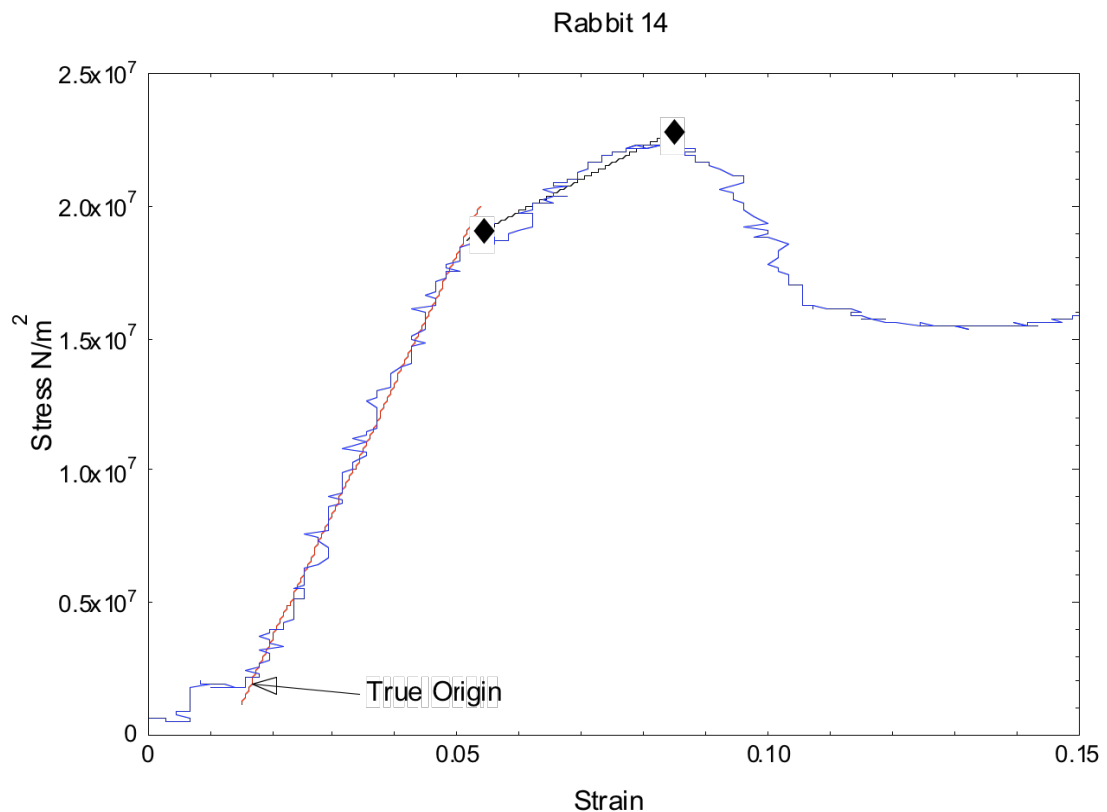


Figure xiii Stress Strain Plot Demonstrating Initial Increase in Strain Without Corresponding Increase in Stress

The data between the yield point and failure point were isolated and a second gradient was produced (Figure xiv). This enabled confirmation of the yield point as evidenced by an obvious alteration in the gradient and therefore defined the extent of the elastic region. The gradient of the linear region was calculated using the curve fitting function of the Easy Plot software. There was an identifiable linear region in all cases and the gradient of each plot was measured on 10 occasions. The mean gradient for each plot was calculated and represented the modulus of elasticity.

This was potentially subject to observer error and for this reason, each curve was assessed in sequence and the process repeated ten times (Figure xv). This prevented observer familiarity with an individual plot and was considered to be the most objective method of estimating the relevant points. The yield stress and strain, and failure stress and strain were also calculated in this manner. All data were entered into the Stats Direct Statistical Software programme and saved for individual specimens and as a cumulative data file.

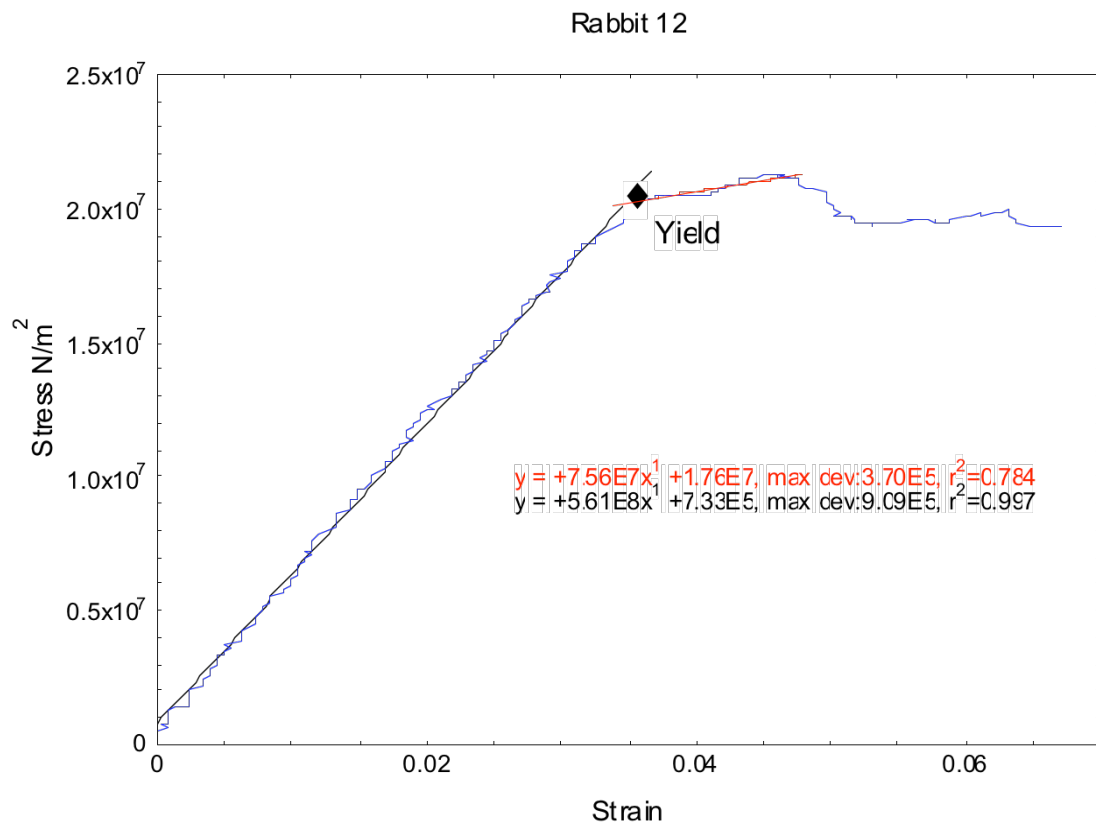


Figure xiv Determination of Modulus of Elasticity

Analysis was performed for all tibiae and individual measurements were combined to form a single data set for each of the four groups.

Group 1 Chemotherapy

Group 1 Control

Group 2 Chemotherapy

Group 2 Control

This produced a range, mean, and standard deviation for the following parameters in each group:

Modulus of elasticity	N/m ²
Energy to yield	J
Yield stress	N/m ²
Yield strain	-
Energy to failure	J
Failure stress	N/m ²
Failure strain	-

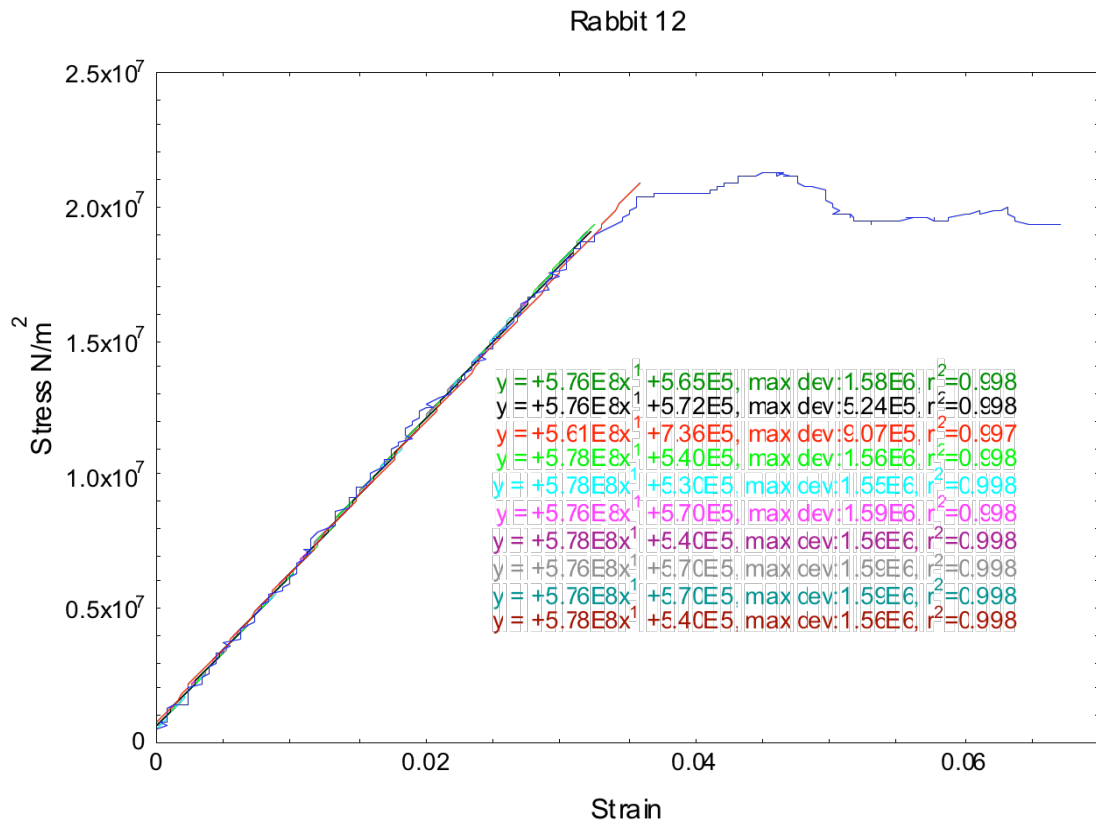


Fig xv

3.2.5 Statistical Analysis

Data from all sources were saved as an Excel spreadsheet. This interfaced with Stats Direct statistical software package, which was used for all statistical analysis. The statistical requirements for this project were discussed with Dr. Hazel Taylor (University of Bristol) and the following approach was recommended.

It was initially assumed that the data was normally distributed and this was confirmed using a Shapiro Wilk test. In all cases, data was from a population with normal distribution.

Descriptive statistics are presented as Box and Whisker plots demonstrating the mean, standard deviation, minimum and maximum for each parameter.

An unpaired t-test was used to compare groups and this was prefaced by an F-test to confirm equal variance. The two-sided statistic with a 95% confidence interval was used in each case

Statistical significance was assumed at the $p=0.05$ level and power for this level of significance was also determined by *post hoc* analysis.

3.3 Regenerate Parameters

3.3.1 Length / mm

		Mean	σ
Neo-adjuvant	Control	0.74	0.15
	Chemotherapy	0.73	0.11
Adjuvant	Control	0.66	0.13
	Chemotherapy	0.72	0.08

Neo-adjuvant

$p = 0.84$

95% confidence interval for difference between means = -0.11 to 0.13

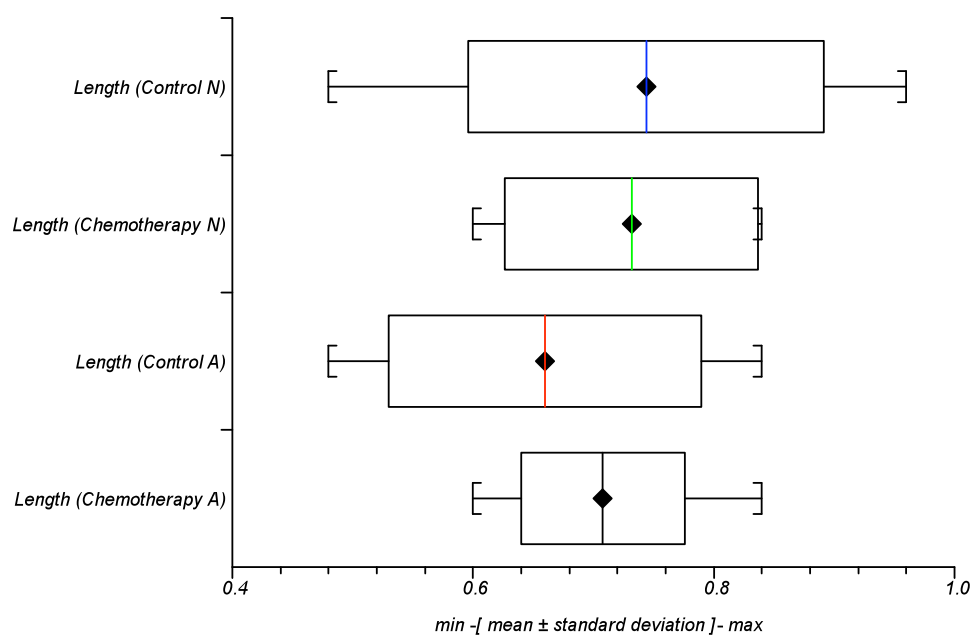
Power (for 5% significance) = 5.88%

Adjuvant

$p = 0.31$

95% confidence interval for difference between means = -0.15 to 0.05

Power (for 5% significance) = 28.06%



3.3.2 Area / cm²

		Mean	σ
Neo-adjuvant	Control	0.77	0.29
	Chemotherapy	0.57	0.06
Adjuvant	Control	0.54	0.14
	Chemotherapy	0.49	0.15

Neo-adjuvant

F test significant therefore assuming unequal variances

p = 0.06

95% confidence interval for difference between means = 5.4×10^{-3} to 0.4

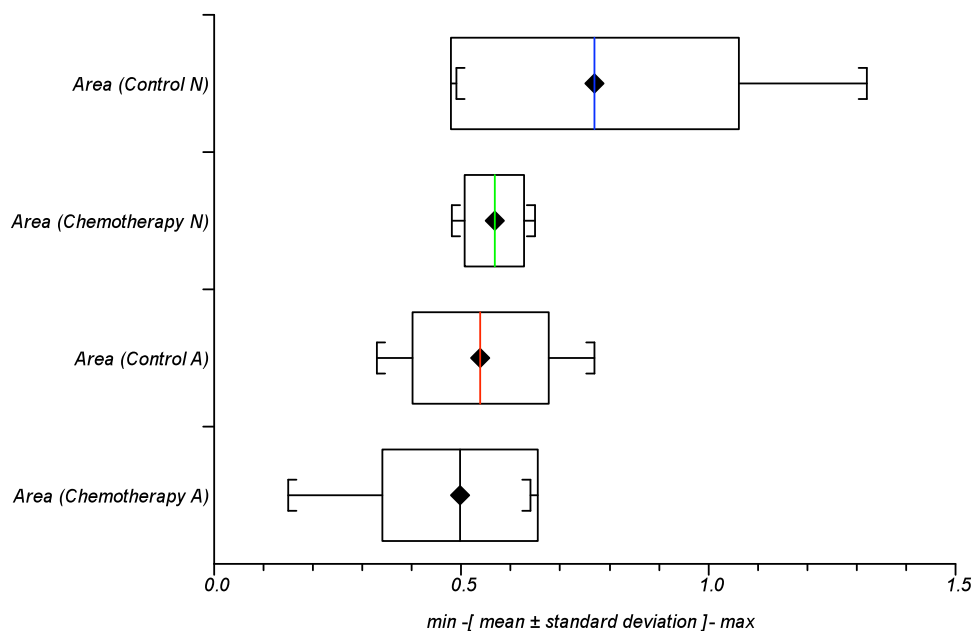
Power (for 5% significance) = 46.98%

Adjuvant

p = 0.53

95% confidence interval for difference between means = -0.10 to 0.18

Power (for 5% significance) = 13.38%



3.3.3 Volume/cm³

		Mean	σ
Neo-adjuvant	Control	0.58	0.27
	Chemotherapy	0.42	0.07
Adjuvant	Control	0.35	0.12
	Chemotherapy	0.35	0.09

Neo-adjuvant

F test significant therefore assuming unequal variances;

$p = 0.10$

95% confidence interval for difference between means = -0.03 to 0.35

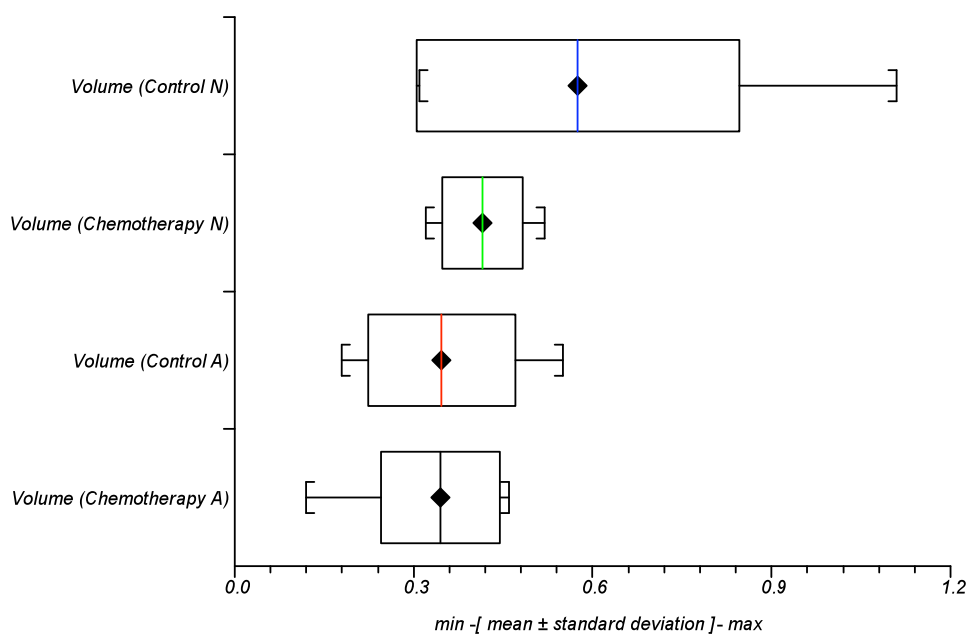
Power (for 5% significance) = 34.49%

Adjuvant

$p = 0.97$

95% confidence interval for difference between means = -0.10 to 0.11

Power (for 5% significance) = 5.03%



3.3.4 Oblique Plane Alignment (°)

		Mean	σ
Neo-adjuvant	Control	3.62	3.73
	Chemotherapy	5.85	3.10
Adjuvant	Control	5.73	1.66
	Chemotherapy	4.52	2.18

Neo-adjuvant

$p = 0.22$

95% confidence interval for difference between means = -5.04 to 1.23

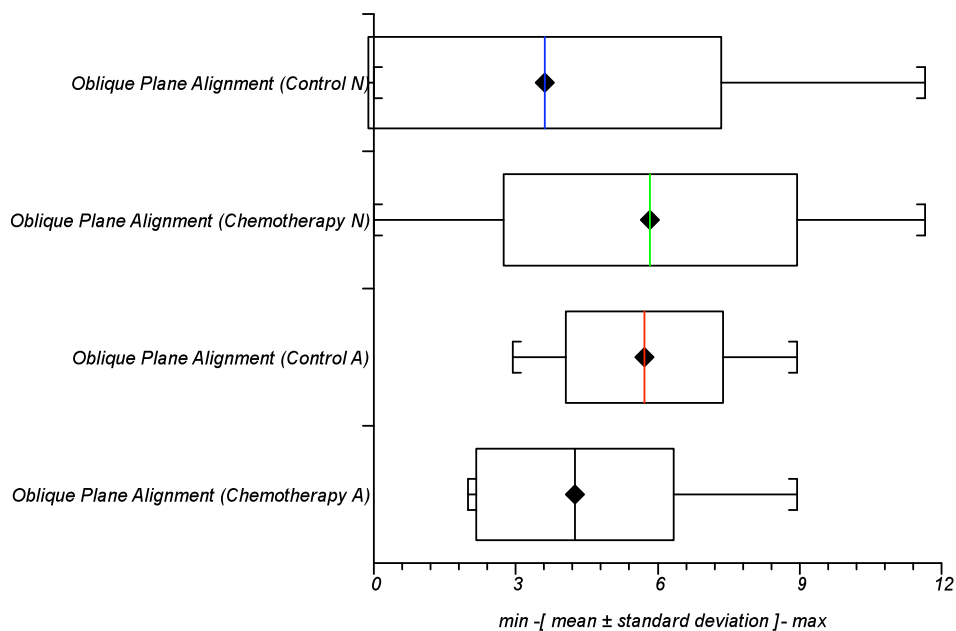
Power (for 5% significance) = 41.17%

Adjuvant

$p = 0.17$

95% confidence interval for difference between means = -0.57 to 2.99

Power (for 5% significance) = 45.93%



3.4 DXA Parameters

3.4.1 Bone Mineral Content

		Mean	σ
Neo-adjuvant	Control	0.36	0.14
	Chemotherapy	0.24	0.04
Adjuvant	Control	0.22	0.08
	Chemotherapy	0.23	0.06

Neo-adjuvant

F test significant therefore assuming unequal variances;

$p = 0.02$

95% confidence interval for difference between means = 0.03 to 0.23

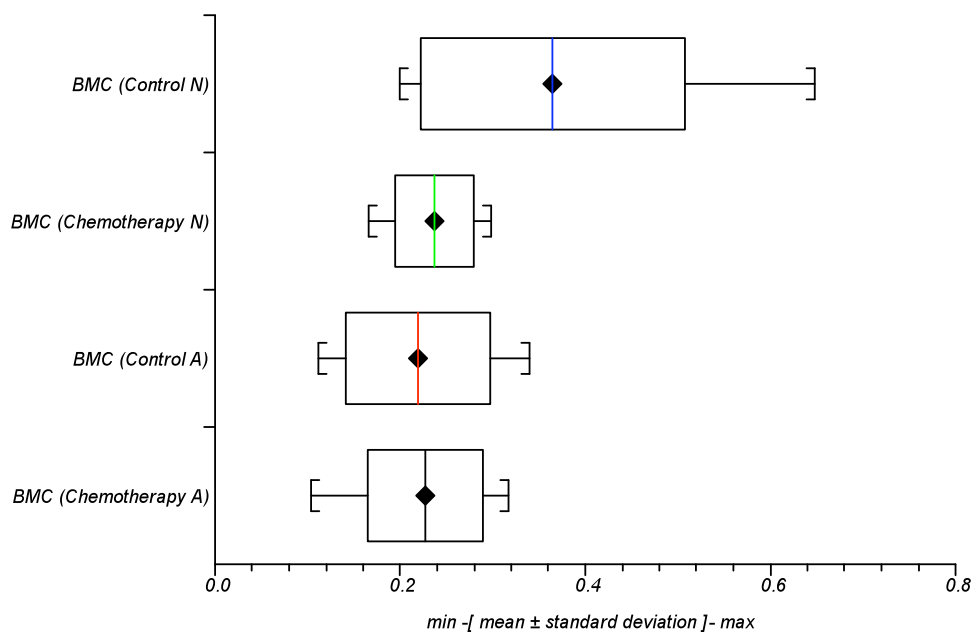
Power (for 5% significance) = 68.41%

Adjuvant

$p = 0.79$

95% confidence interval for difference between means = -0.07 to 0.06

Power (for 5% significance) = 6.47%



3.4.2 Bone Mineral Density

		Mean	σ
Neo-adjuvant	Control	0.50	0.07
	Chemotherapy	0.38	0.04
Adjuvant	Control	0.40	0.06
	Chemotherapy	0.40	0.06

Neo-adjuvant

$p = < 0.01$

95% confidence interval for difference between means = 0.06 to 0.17

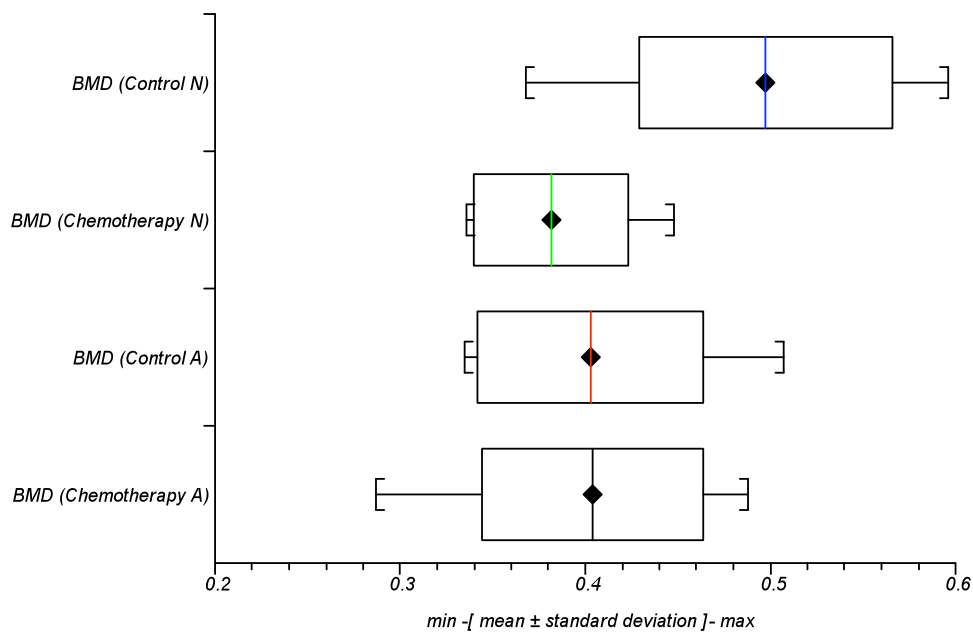
Power (for 5% significance) > 99.99%

Adjuvant

$p = 0.97$

95% confidence interval for difference between means = -0.06 to 0.05

Power (for 5% significance) = 5.03%



3.4.3 Volumetric Bone Mineral Density

		Mean	σ
Neo-adjuvant	Control	0.65	0.09
	Chemotherapy	0.57	0.06
Adjuvant	Control	0.63	0.05
	Chemotherapy	0.67	0.09

Neo-adjuvant

$p = 0.04$

95% confidence interval for difference between means = 0.006 to 0.15

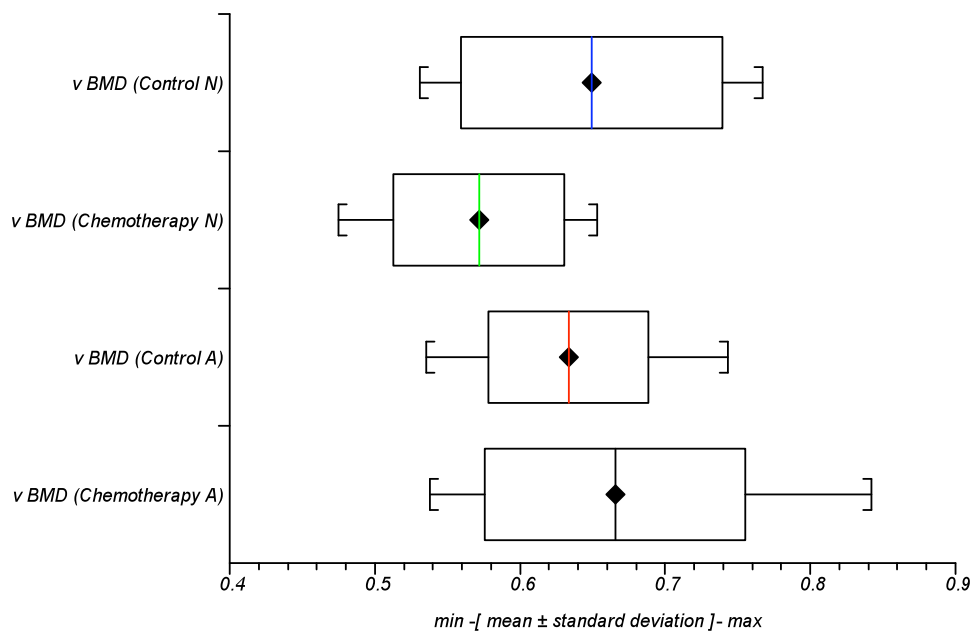
Power (for 5% significance) = 86.13%

Adjuvant

$p = 0.34$

95% confidence interval for difference between means = -0.10 to 0.04

Power (for 5% significance) = 24.86%



3.5 Mechanical Parameters

3.5.1 Modulus of Elasticity

		Mean	σ
Neo-adjuvant	Control	5.88×10^8	3.52×10^8
	Chemotherapy	5.92×10^8	1.15×10^8
Adjuvant	Control	4.62×10^8	1.41×10^8
	Chemotherapy	5.65×10^8	2.84×10^8

Neo-adjuvant

F test significant therefore assuming unequal variances;

$p = 0.97$

95% confidence interval for difference between means = -2.62 to 2.44×10^8

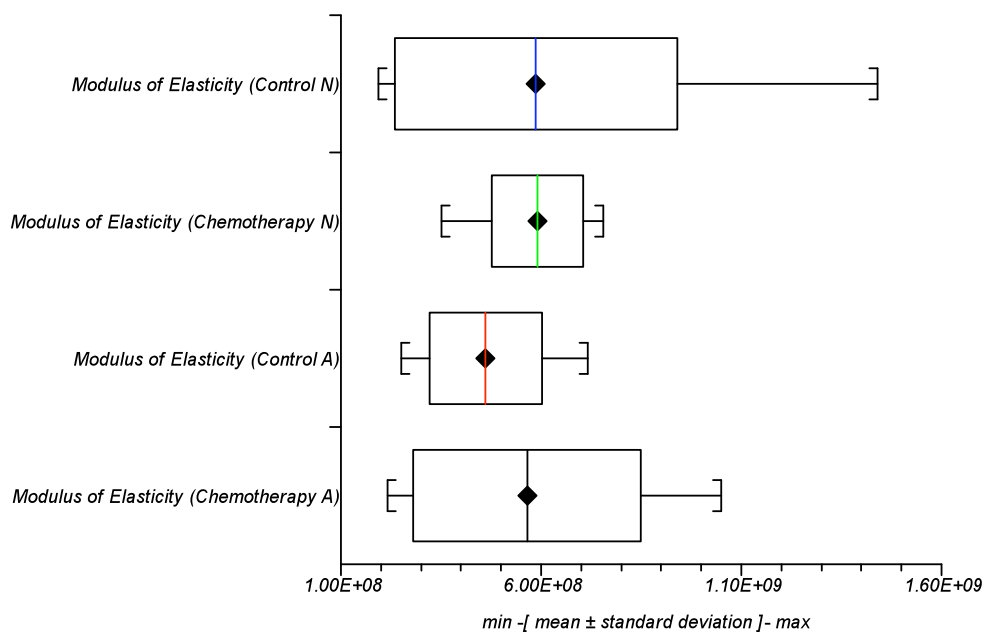
Power (for 5% significance) = 2.63%

Adjuvant

$p = 0.32$

95% confidence interval for difference between means = -3.1 to 1.1×10^8

Power (for 5% significance) = 26.07%



3.5.2 Energy at Yield

		Mean	σ
Neo-adjuvant	Control	3.14×10^7	1.74×10^7
	Chemotherapy	2.46×10^7	1.07×10^7
Adjuvant	Control	3.93×10^7	6.26×10^7
	Chemotherapy	2.74×10^7	1.22×10^7

Neo-adjuvant

$p = 0.31$

95% confidence interval for difference between means = -6.99×10^6 to 2.07×10^7

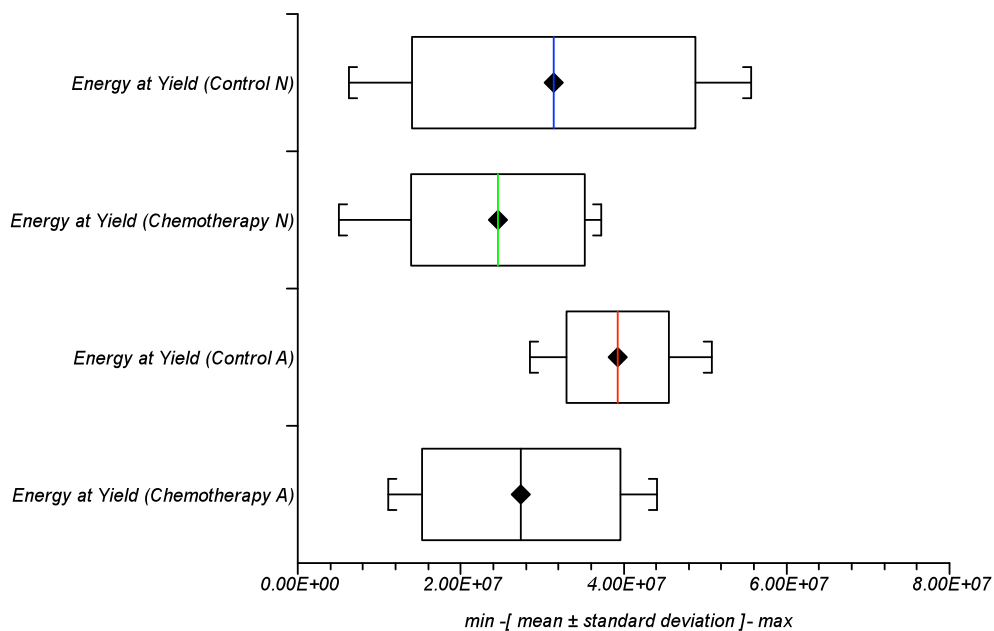
Power (for 5% significance) = 13.69%

Adjuvant

$p = 0.01$

95% confidence interval for difference between means = 2.64×10^6 to 2.11×10^7

Power (for 5% significance) = 94.13%



3.5.3 Yield Stress

		Mean	σ
Neo-adjuvant	Control	1.63×10^6	5.40×10^6
	Chemotherapy	1.64×10^6	3.40×10^6
Adjuvant	Control)	1.89×10^7	2.38×10^7
	Chemotherapy	1.68×10^7	6.49×10^7

Neo-adjuvant

$p = 0.94$

95% confidence interval for difference between means = -4.47 to 4.16×10^6

Power (for 5% significance) = 5.11%

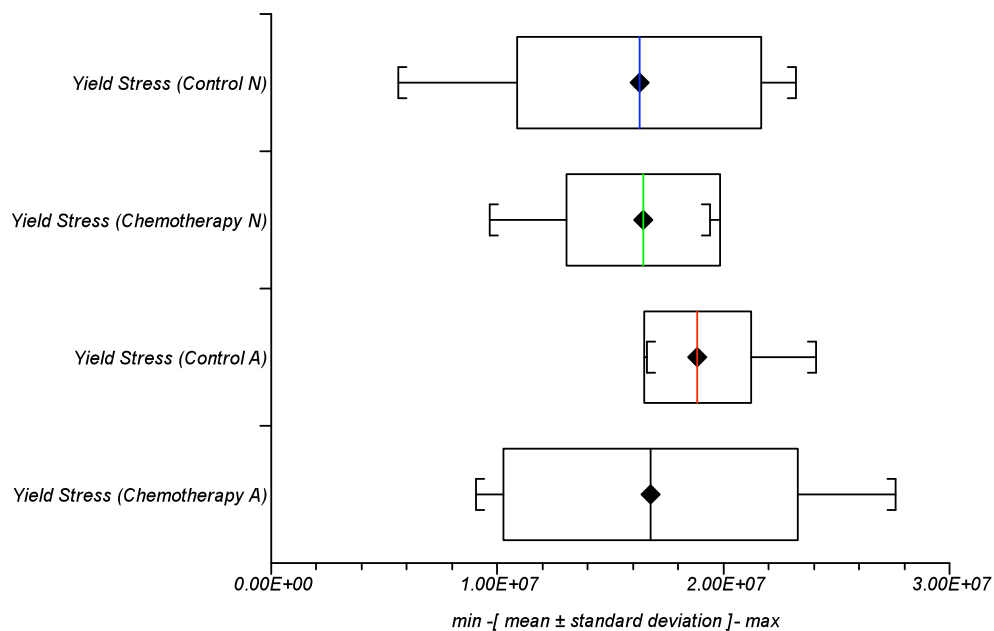
Adjuvant

F test significant therefore assuming unequal variances;

$p = 0.39$

95% confidence interval for difference between means = -2.8 to 6.9×10^6

Power (for 5% significance) = 10.79%



3.5.4 Yield Strain

		Mean	σ
Neo-adjuvant	Control	3.68×10^{-2}	1.49×10^{-2}
	Chemotherapy	3.22×10^{-2}	1.02×10^{-2}
Adjuvant	Control	5.20×10^{-2}	1.35×10^{-2}
	Chemotherapy	3.68×10^{-2}	1.01×10^{-2}

Neo-adjuvant

$p = 0.44$

95% confidence interval for difference between means = -7.0×10^{-3} to 1.7×10^{-2}

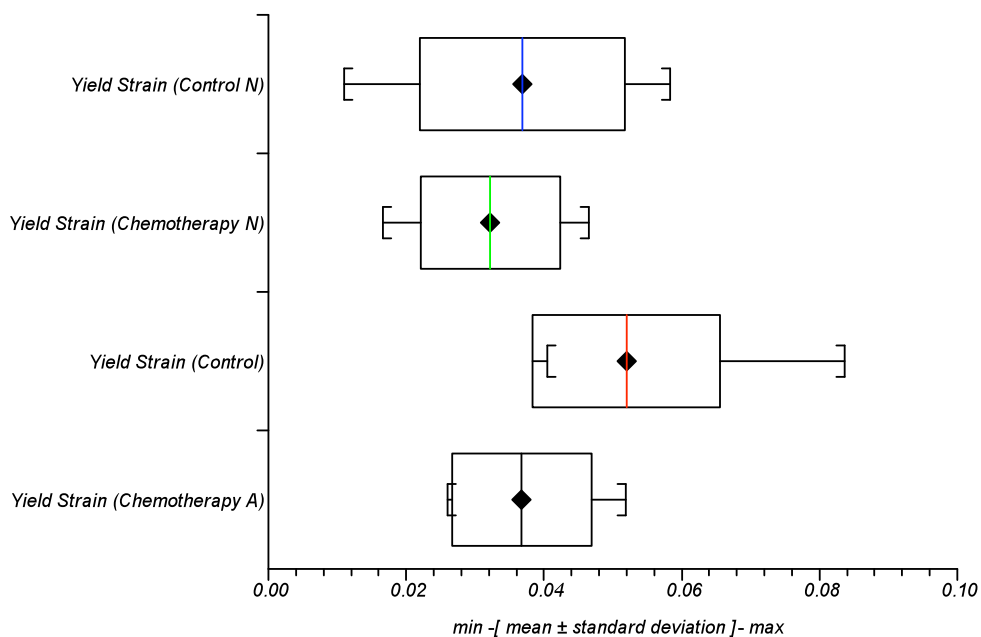
Power (for 5% significance) = 19.04%

Adjuvant

$p = 0.01$

95% confidence interval for difference between means = 2.7×10^{-2} to 3.5×10^{-2}

Power (for 5% significance) = 94.55%



3.5.5 Energy at Failure

		Mean	σ
Neo-adjuvant	Control	5.48×10^7	2.33×10^7
	Chemotherapy	8.09×10^7	9.75×10^7
Adjuvant	Control	6.41×10^7	2.28×10^7
	Chemotherapy	5.25×10^7	2.07×10^7

Neo-adjuvant

$p = 0.59$

95% confidence interval for difference between means = -1.60×10^7 to 2.73×10^8

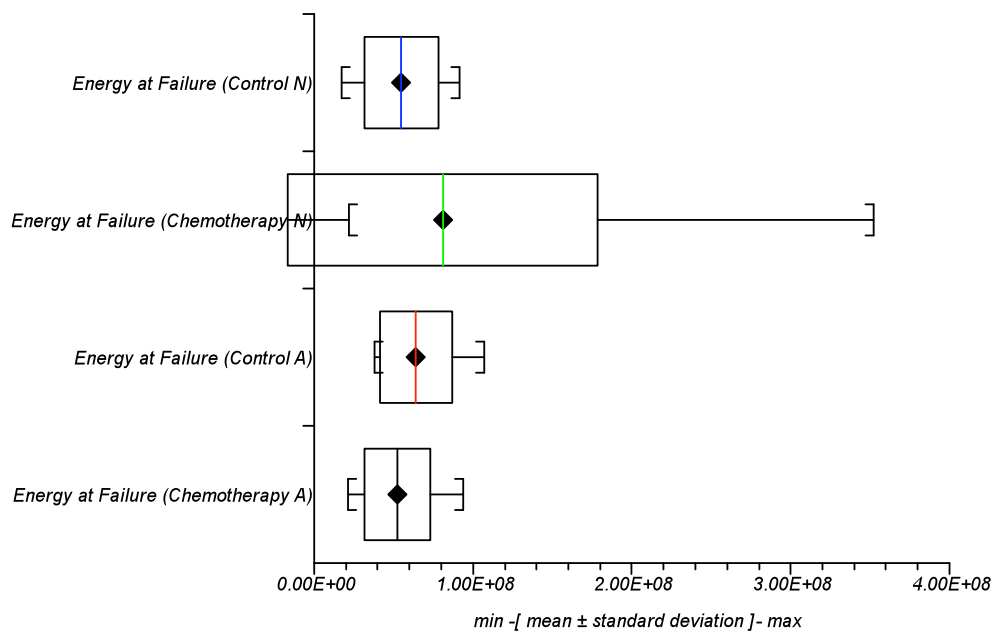
Power (for 5% significance) = 6.76%

Adjuvant

$p = 0.27$

95% confidence interval for difference between means = -9.5×10^6 to 3.36×10^7

Power (for 5% significance) = 67.78%



3.5.6 Failure Stress

		Mean	σ
Neo-adjuvant	Control)	1.87×10^7	5.52×10^7
	Chemotherapy)	1.95×10^7	3.52×10^7
Adjuvant	Control	1.98×10^7	2.73×10^7
	Chemotherapy)	1.88×10^7	6.30×10^7

Neo-adjuvant

$p = 0.71$

95% confidence interval for difference between means = -5.23 to 3.64×10^6

Power (for 5% significance) = 4.86%

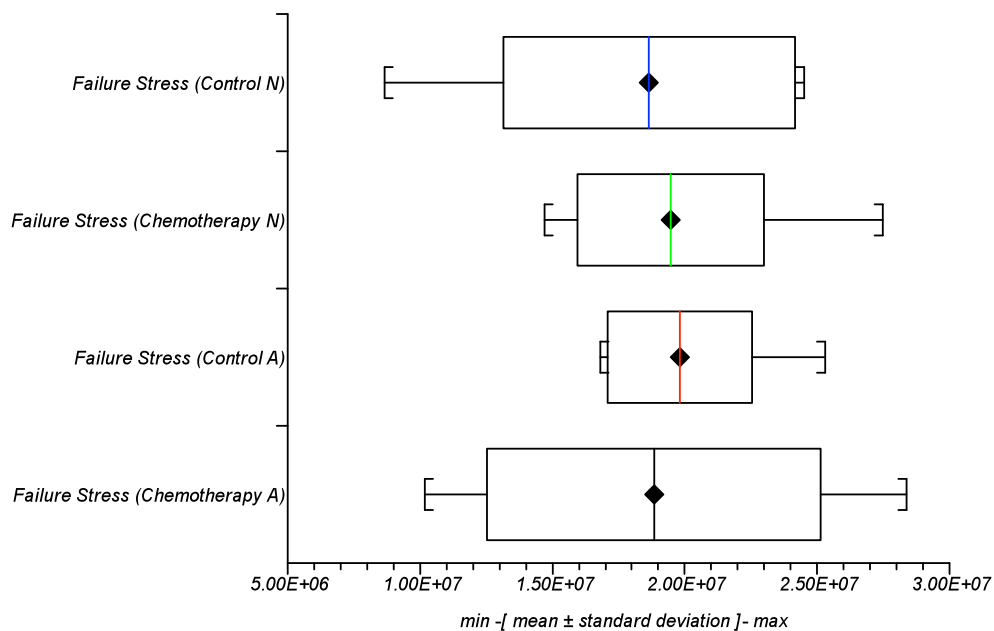
Adjuvant

F test significant therefore assuming unequal variances;

$p = 0.67$

95% confidence interval for difference between means = -3.8×10^6 to 5.8×10^6

Power (for 5% significance) = 5.18%



3.5.7 Failure Strain

		Mean	σ
Neo-adjuvant	Control)	5.36×10^{-2}	1.88×10^{-2}
	Chemotherapy	4.74×10^{-2}	1.33×10^{-2}
Adjuvant	Control	6.39×10^{-2}	1.29×10^{-2}
	Chemotherapy	5.24×10^{-2}	1.71×10^{-2}

Neo-adjuvant

$p = 0.41$

95% confidence interval for difference between means = -1.0×10^{-2} to 2.2×10^{-2}

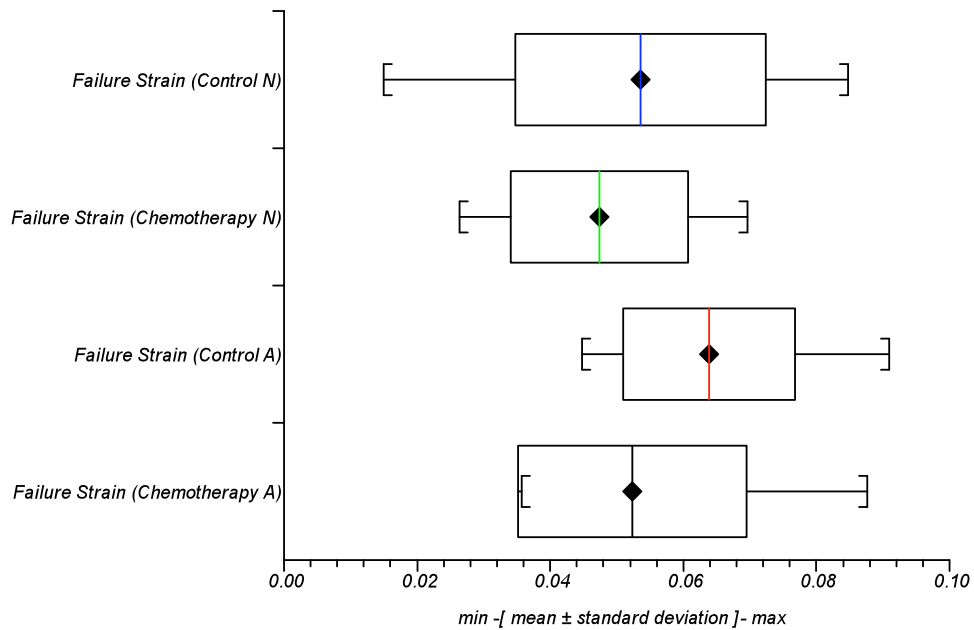
Power (for 5% significance) = 20.21%

Adjuvant

$p = 0.11$

95% confidence interval for difference between means = -3.0×10^{-3} to 2.6×10^{-2}

Power (for 5% significance) = 58.1%



3.6 Significant Adverse Events

3.6.1 Control Groups

Rabbit 1

On day 4, the animal was noted to be dragging the left hock but did not appear distressed. The limb was inspected by the Veterinarian without anaesthesia and foot appeared to be flail, without external signs of injury and with normal perfusion. There was no imperative to explore the limb or terminate the experiment and normal limb function was observed by the time of the second infusion.

Rabbit 3

A significant (> 0.5 cm), non-infected medial wound dehiscence was identified on day 10 and was resutured under general anaesthesia. The wound was healing at the time of the second infusion on day 14 and did not require any further intervention.

Rabbit 5

A significant, non-infected medial wound dehiscence was identified on day 8 and was resutured under general anaesthesia. The wound had healed by the time of the second infusion on day 14 and did not require any further intervention.

Rabbit 7

A significant, non-infected medial wound dehiscence was identified on day 15 and was resutured under general anaesthesia. The wound did not require any further intervention and had healed by day 28.

Rabbit 8

A significant, non-infected wound dehiscence was identified on day 2 and was resutured under general anaesthesia. There was a further dehiscence on day 4 requiring excision of infected, necrotic wound edges and resuturing. On day 14, there was a smooth induction of anaesthesia but prior to surgical exposure of the left groin, the animal became apnoeic. It was noted that there was copious turbid fluid in the mouth and the *post mortem* examination demonstrated that the lungs were congested with turbid fluid suggesting an aspiration pneumonitis.

Rabbit 9

A minor (< 0.5cm), non-infected medial wound dehiscence was noted during pre-anaesthetic assessment on day 14 but this did not require treatment.

On day 16 there was a significant non-infected wound dehiscence that required resuturing under anaesthesia. This subsequently healed without further intervention.

On day 17, a raw area was noted over the right knee, probably due to contact with the cage. This was inspected under general anaesthesia and the knee was padded with Betadine gauze and dressed with Elastoplast. This was reinspected under anaesthesia at the time of osteotomy on Day 28, and was noted to have healed without infection.

Rabbit 11

The initial inguinal exposure, cannulation and infusion were uncomplicated.

The animal became apnoeic during the second saline infusion.

Post mortem examination demonstrated aspiration pneumonitis which was considered to be due to general anaesthesia without endotracheal intubation. .

3.6.2 Chemotherapy Groups

Rabbit F

The animal developed a partial thickness abrasion to the right proximal medial thigh on Day 1 due to contact with the metal cage. This was inspected and irrigated with sterile saline without anaesthesia for 3-consecutive days. The abrasion healed completely within seven days.

Rabbit O

A significant non-infected dehiscence was identified on day 3 and was resutured under general anaesthesia. The wound did not require any further intervention and had healed by day 14.

3.7 Technical Failures

3.7.1 Control Groups

Rabbit 1

The tibia was damaged during transit to the Department of Mechanical Engineering and was not suitable for testing.

Rabbit 2

The disarticulated hock was lost in transit between the Animal Care Facility and the Department of Radiology and destroyed without further examination

Rabbit 5

The disarticulated hock was lost in transit between the Animal Care Facility and the Department of Radiology and destroyed without further examination

3.7.2 Chemotherapy Groups

Rabbit BB

The tibia was incorrectly mounted with insufficient tibia between the resin mounting blocks. The bone was subjected to compression testing but this did not produce any meaningful data.

Rabbit L

The disarticulated hock was lost in transit between the Animal Care Facility and the Department of Radiology and destroyed without further examination

Rabbit P

The disarticulated hock was lost in transit between the Animal Care Facility and the Department of Radiology and destroyed without further examination

Rabbit X

The tibia was incorrectly mounted with insufficient tibia between the resin mounting blocks. The bone was subjected to compression testing but this did not produce any meaningful data.

3.8 Summary of Results

The raw experimental data is presented in table form. (Appendix 4)

There were no statistically significant differences in regenerate length, area, volume and alignment between chemotherapy and control groups in either arm of the experiment.

There was a statistically significant reduction in BMC, BMD and ν BMD in the chemotherapy group of the neo-adjuvant arm of the experiment.

There were no statistically significant differences in the DXA parameters in the adjuvant arm of the experiment.

There were no statistically significant differences in mechanical properties between groups in the neo-adjuvant arm.

In the adjuvant arm, there was a statistically significant increase in energy at yield and yield strain in the control group.

The relevance of these data and the implications for the clinical use of distraction osteogenesis are considered in the following section.

Section 4 Discussion, Clinical Relevance and Concluding Remarks

The purpose of this work was to investigate the effect of cis-platinum and adriamycin on regenerate bone formation in a juvenile NZW rabbit model of distraction osteogenesis. The clinical basis was to explore the feasibility of limb salvage using bone transport following surgical excision of an osteogenic sarcoma, in the presence of neo-adjuvant or adjuvant cytotoxic drugs.

A systematic review of the literature describing bone transport in the clinical context did not identify a consistent detrimental effect on bone formation. The relevant literature however, considered small patient numbers in uncontrolled series. It was not possible to reach any robust conclusions and this study attempted to address the knowledge gap. Confirmation that the structural and material properties of the regenerate were unaffected by the cytotoxic agents would support the use of distraction osteogenesis for tumour reconstruction.

The experiment consisted of two separate pillars of study. It was initially necessary to develop a reproducible animal model that simulated the metabolic effects of cyclical cytotoxic chemotherapy, without the confounding effects of renal and hepatic failure. This model was used to evaluate the effects of these agents on the material and structural properties of regenerate bone formed by distraction osteogenesis, which was used as a surrogate for bone transport.

4.1 Development of the Animal Model

This component of the experiment defined an appropriate cytotoxic regimen, which fulfilled the conflicting criteria of a clinically relevant response demonstrated by an appropriate effect on body weight, without biochemical evidence of renal or hepatic failure.

The use of single animal data is a deficiency of this study and an increased number of animals in each dose group would enhance data interpretation. This data however, provided an indicator of an appropriate dose and it was a pragmatic approach, which was possible within the time constraints and resources available for the study.

The initial component of this study investigated the effects of 2 cycles of cis-platinum and adriamycin, infused with an IVAC pump over 2-hours, at an interval of 14-days using various dose combinations. The infusion technique involved insertion of a peripheral cannula into the femoral vein and this technique was refined in the initial experimental group.

It was possible to predictably identify the vascular anatomy and isolate the femoral vein using sling sutures. It was necessary to produce watertight cannulation of the vein with a single attempt to prevent contamination of the adjacent soft tissues, which would introduce a significant risk of soft tissue necrosis. In all cases, it was possible to identify and cannulate the femoral vein, with uncomplicated delivery of drugs.

A pre-requisite of the model was the production of an adequate cytotoxic effect and the initial component of the experiment involved the development of a model that predictably produced a reduction in percentage weight gain, whilst preserving normal renal and hepatic function, assessed by analysis of peripheral blood samples. Renal or hepatic failure following cytotoxic infusion would introduce a confounding variable and any subsequent effect on bone formation could not be confidently attributed to the cytotoxic drugs.

Infusion with 6.5 mg/kg cis-platinum and 6.5 mg/kg adriamycin led to failure to gain weight with a rapid deterioration in renal function and general condition 8-days after infusion, with death on day 11. Frank *et al* (Frank et al., 1989) investigated the effect of 8 mg/kg cis-platinum and demonstrated MRI evidence of nephrotoxicity within 9-hours with significant deterioration of renal function (BUN, creatinine). Histology from this series demonstrated mild to moderate focal necrosis of the proximal convoluted tubule. Histological analysis was not available for this experiment, but the macroscopic *post mortem* findings included enlargement of both kidneys, granular renal parenchyma and an increase in perirenal fat. This suggested that although the dose of cis-platinum was lower, combination with an equivalent dose of adriamycin produced a rapidly fatal deterioration in general condition and renal function.

Infusion with 4.5 mg/kg cis-platinum and 4.5 mg/kg adriamycin led to an initial reduction in the rate of weight gain and the pre-infusion weight did not recover until

26-days after the initial dose. This would have been ideal in terms of the cytotoxic effect but there was a rapid increase in urea and creatinine, peaking approximately 2-4-days after each infusion. At the conclusion, there was persistent elevation of serum creatinine (164 μ mol/L) and serum urea (13.4 mmol/L).

The pattern of renal failure mirrors that described by Najjar *et al* (Najjar and Saad, 2001) after an infusion of 5 mg/kg with a highly significant increase in serum creatinine and urea within 7-days of infusion.

Persistent abnormalities in renal function would potentially influence bone mineralisation due to the effect on calcium and phosphate homeostasis, parathyroid hormone and vitamin D metabolism. This would introduce an important confounding variable and make subsequent data interpretation difficult. A fundamental requirement of the experimental model was therefore preservation of renal function.

Reduction to 3 mg/kg cis-platinum and 3 mg/kg adriamycin resulted in an initial 11% and subsequent 13% loss of percentage body weight but led to an increase in urea and creatinine within 3-days of the initial infusion. This approximates the lower dose group described by Frank *et al* (Frank *et al.*, 1989) and although the total dose received was greater, the individual doses are identical. There was a catastrophic deterioration in renal function leading to death at day-21 suggesting that the combined effect of the 2 cytotoxic agents produced an amplified nephrotoxic effect although this was not validated by histological examination.

Further reduction to 2 mg/kg cis-platinum and 2 mg/kg adriamycin produced a more modest reduction in weight gain, returning to the pre-infusion weight 8-days after initial infusion, with a gradual increase in weight and a small decline after the second infusion. There was an initial increase in serum creatinine and urea, which was also maximum 2-4 days after initial infusion, suggesting renal tubular damage and recovery. After the second infusion there was a profound increase in serum creatinine with a modest increase in serum urea, which persisted until the conclusion of the experiment. The haematocrit during this period was unrelated to urea and creatinine levels. This suggested acute tubular damage following infusion with a cumulative effect of both agents on the degree of tubular damage and rate of recovery.

Two cycles of 1 mg/kg cis-platinum and 1 mg/kg adriamycin over 2-hours did not adversely affect renal function. The percentage weight loss however was small and was regained by day 6 with an increase in percentage body weight thereafter. This would potentially lead to the conclusion that any lack of observed alteration in the properties of the regenerate would be due to sub-therapeutic doses of the agents used.

The compromise was an initial infusion of 1 mg/kg cis-platinum and 2mg/kg adriamycin, increasing to 4 mg/kg and this was used for the main component of the experiment. The aim was to prevent the deterioration in renal function associated with doses of cis-platinum greater than 1 mg/kg, minimise the risk of the amplified deterioration associated with these drugs in combination and produce a clinically relevant cytotoxic effect. This schedule acknowledged concerns relating idiosyncratic sensitivity to adriamycin. (Bocherens-Gadient et al., 1992) It was necessary to promptly identify animals that were particularly sensitive and replace early in the experiment and before application of external fixator and osteotomy.

The timing of drug administration was also investigated and the schedules were broadly defined as neo-adjuvant if drugs were given before surgery and adjuvant if additional drugs were given after surgery. Both strategies are used in the management of osteogenic sarcoma and the model attempted to mimic the COSS (Link et al., 1991, Link et al., 1986) and EOI (Bramwell et al., 1992, Souhami et al., 1997) approach to this clinical problem.

This formed the basis of the cytotoxic chemotherapy schedule for the distraction osteogenesis experiment. The drugs were given 4 and 2-weeks before osteotomy and distraction (Neo-adjuvant Group) or 10-days before and 4-days after osteotomy and distraction (Adjuvant Group).

4.2 Validation of the Animal Model

Anaesthesia and surgical procedures were well tolerated in the majority of cases. The animals recovered rapidly, did not appear distressed and were observed until alert and drinking before being returned to their cage. An animal care assistant monitored the animals on a daily basis and significant adverse events were

documented, discussed with the Veterinarian and a decision made on continuing with the experiment or sacrificing the animal.

In the early part of the experiment, general anaesthesia was complicated by fatal aspiration pneumonitis during induction of anaesthesia in 2 cases. Diet was therefore restricted for 2-hours before infusion with clear fluids continued *ad libitum* and this complication was eradicated.

Consideration was given to pooling the control data for the neo-adjuvant and adjuvant groups to produce a single control group. This would potentially simplify the presentation of results and allow direct comparison of active groups.

Preliminary statistical analysis demonstrated significant differences between the control data for several parameters and invalidated this approach. This observation confirmed the need for a properly controlled environment and individual data is presented for each group.

4.3 Neo-adjuvant Chemotherapy Group

The active group received 1.0 mg/kg cis-platinum and 2.0 mg/kg adriamycin at 8-weeks and 1.0 mg/kg cis-platinum and 4.0 mg/kg adriamycin at 10-weeks. The tibial osteotomy was performed when the animals were 12-weeks old.

Rabbits that had been infused with cis-platinum and adriamycin demonstrated a statistically significant reduction in relative weight gain at 10, 12 and 16-weeks of age. Decrease in weight has been identified in previous animal studies as an indicator of cytotoxic effect. (Wanless et al., 1987) This effect is also seen in human clinical practice and is due to several factors, including anorexia and a catabolic response to cytotoxic drugs. The difference demonstrated in each experimental group suggests that the animals received clinically relevant amounts of cis-platinum and adriamycin. It was not possible to quantify the mechanism leading to this observation, as food and water were given *ad libitum*.

There were significant differences in the serum haemoglobin between groups at the start of the experiment, this trend had reversed at the time of the tibial osteotomy and there was no observable difference in haemoglobin concentration at the conclusion of the experiment.

There is no obvious explanation for the initial difference. These animals were of equivalent age, reared in identical conditions and there was no evidence of concomitant disease. The specimens were provided and selected without any known bias. The animals were not randomised but there was no consistently observed difference in the clinical condition, weight or other features.

The reversal in trend at the time of tibial osteotomy suggests that haematopoiesis was affected by the cytotoxic infusion, which has been demonstrated in previous studies using adriamycin in this species. (Wanless et al., 1987) The relative anaemia following chemotherapy is similar to the effect of therapeutic doses of cytotoxic agents in human patients. This is due in part to marrow suppression and whilst it suggests that the animal model was clinically relevant, it has not been directly demonstrated. Future work should include marrow biopsy to investigate whether the alteration in haemoglobin concentration occurs as a result of marrow suppression

There was no effect on the leucocyte count at any of the points of measurement. The pattern of leucopenia seen in the dose response group indicated that the decline in leucocyte count was most obvious between 2 and 4-days after infusion and the lack of observed effect may have been due to the timing of the samples. It could also indicate a lack of therapeutic effect, but in the presence of decline in haemoglobin and weight gain in the chemotherapy groups, this was considered less likely. Future work should assess leucocyte count at intervals of 24-48 hours to determine whether there is a transient reduction following cytotoxic infusion. This should be correlated with an alteration in marrow composition to confirm that the model was a reasonable surrogate.

There was a reduction of total protein concentration at 12-weeks (osteotomy) in the chemotherapy group, but the difference in means was small (58.4 g/L vs. 52.9 g/L) with a minimum in each group of 49.0 g/L. This difference was not considered clinically significant and was not present at the conclusion of the experiment. The stable total protein indicates that hepatic production and renal excretion are unaffected, consistent with normal laboratory tests of hepatic and renal function (*vide infra*). It also implies that the observed weight loss was not due to anorexia and associated malnutrition although there is no direct evidence. The duration of

the experiment was short and conclusions relating to malnutrition would be more accurately addressed by measuring dietary intake.

The combination of decreased weigh gain and reduction in haemoglobin indicates that the chemotherapy regimen adversely affected the general condition of the animals. It demonstrates that the model has merit and produces effects in the rabbit that would be expected in a human patient.

There was a statistically significant increase in serum urea following the initial cytotoxic infusion (10 weeks), which had recovered at the conclusion of the experiment. The difference in means was small (6.32 mmol/L vs. 6.31 mmol/L) and was not considered to be clinically significant.

There was no correlation between urea, creatinine and haematocrit, excluding dehydration as a cause for this observation. There was no statistically significant difference in serum creatinine between groups at any point in the experiment.

A statistically significant increase in ALT was noted at the time of the osteotomy in the control group, with a large difference in means (121.21 U/L vs. 44 U/L). This observation is difficult to explain, as the reverse would be expected given the hepatotoxic properties of adriamycin.

There was statistically significant increase in γ GT in the control group but the difference in means was small (11.30 U/L vs. 6.00 U/L) and all measurements were within the normal limits defined by the initial levels. There was no difference in ALT or γ GT at the conclusion suggesting that there was no prolonged effect on hepatic function.

4.4 Adjuvant Chemotherapy Group

This group received 1.0 mg/kg cis-platinum and 2.0 mg/kg adriamycin at 10-weeks. The tibial osteotomy was performed when the animals were 12-weeks old and 1.0 mg/kg cis-platinum and 4.0 mg/kg adriamycin was infused 4-days later. The interval between infusions was therefore constant at 2-weeks.

Rabbits in the active group demonstrated reduced weight gain at 12 (5.85%) and 16-weeks (6.1%). Each of these differences was statistically significant at the $p =$

0.05 level with a power of 85 and 80% suggesting that, as in the initial group, this was a robust observation.

The haemoglobin levels were initially equal, with a statistically significant reduction in means at the time of osteotomy ($p = 0.01$, power 92%) and second infusion ($p = 0.01$, power >99%) in the animals that received chemotherapy. The reduction in haemoglobin was mirrored by a reduction in haematocrit, which was statistically significant but represented a difference in means of 4 L/L, which was not considered to be clinically important. The trend in weight gain and haemoglobin was identical for the adjuvant and neo-adjuvant groups and is further evidence that the chemotherapy schedule had adversely affected the general condition of the experimental animals.

There was no statistically detectable difference in white count at any point in this group. It was not possible to comment on whether an effect had been missed due to the infrequent sampling and the lack of marrow aspirate prevented evaluation of the effect on the stem cell populations.

There was a statistically significant reduction in total protein at the time of the second infusion with a small difference in means of 55.0 g/L vs. 58.4 g/L at the time of the second infusion ($p < 0.01$, power 96%) and this had normalised at the completion of the experiment.

There was statistically significant difference in mean urea levels at the time of the each infusion and osteotomy in the chemotherapy group. This was not considered to be of clinical significance as the difference in means was small (Initial Infusion; 4.62 mmol/L vs. 5.73 mmol/L, Osteotomy; 4.78 mmol/L vs. 6.5 mmol/L; Second Infusion 5.42 mmol/L vs. 6.62 mmol/L) and all except Rabbit W (Osteotomy; 11.1 mmol/L) were within the normal range, based on the presenting urea in both groups. There was a statistically significant increase in serum creatinine concentration at the time of osteotomy and second infusion. The difference in means was small on each occasion (osteotomy; 67.9 μ mol/L vs. 87.5 μ mol/L, second Infusion 70.5 μ mol/L vs. 82.73 μ mol/L) and no individual reading exceeded the upper limit of normal based on the presenting creatinine concentration for both groups. These differences were therefore not considered to be of clinical significance.

There was a statistically significant increase ($p = <0.01$. power 96%) in the mean ALT level at the time of second infusion (12⁴ weeks) in the control group. The difference in means was 10.9 (55.9 U/L vs. 40.27 U/L) and this was therefore unlikely to be clinically relevant. There was a statistically significant increase in γ GT in the chemotherapy group at the time of the second infusion. The difference in means was also small (6.66 U/L vs. 8.27 U/L) and all readings were within the normal limits defined by initial readings suggesting that these differences were unlikely to represent a clinically significant deterioration in hepatic function. In addition, compared to the presenting serum levels, ALT and γ GT were not raised at the conclusion of the experiment demonstrating that any effect on hepatic function was transient.

4.5 Summary

4.5.1 Animal Model

The final model was based on results from a small series of individual rabbits, which were used to estimate the appropriate dose of adriamycin and cis-platinum. This strategy was born out of financial and time constraints and carried an inherent risk of failure.

The prescription however, satisfied the requirements of the experiment and produced a statistically significant reduction in percentage weight gain whilst preserving hepatic and renal function. The technical aspects of the model including anaesthesia and cannulation were conducted without difficulty. It was necessary to restrict diet prior to surgery, but no other modifications were necessary and the model provided the basis for investigation of the second pillar of this thesis.

A more comprehensive investigation involving larger numbers and more sophisticated drug combinations may lead to improvements in the model and should be considered as a precursor to further work on this subject.

A standard surgical technique was developed and used throughout the experiment and involved a mid-diaphyseal osteotomy, with lengthening produced by a mono-lateral external fixator. The rabbit tibia is brittle at this age but it was possible to predictably insert 4 external fixator pins using a standard jig. The osteotomy was

performed with a bone cutter after pre-drilling and this produced a predicable osteotomy, without propagation into the adjacent pins.

Particular care was taken to maintain a sterile surgical field at all times. The wound was irrigated with sterile saline throughout the procedure and was dressed with povidone Iodine following attachment of the fixator.

For the purpose of this experiment, major wound dehiscence was defined as a breakdown greater than 0.5 cm requiring re-suturing under general anaesthetic. The incision was approximately 2 cm in the first part of the experiment and this was considered to contribute to a 30% rate of major dehiscence. As the experiment continued and the vascular anatomy could be identified with more confidence, the surgical wound was reduced to approximately 1cm. This led to a reduction in the number of wound complications, which fell to 2.5% for the subsequent three groups.

The tibial osteotomy was performed at 12-weeks of age in an attempt to reproduce the stage of skeletal development equivalent to a human adolescent. This was based on the age of physeal closure as previously reported by Kawblum. (Kaweblum et al., 1994) It was possible to lengthen the right tibia an average of 7.1 mm, which is approximately 10% of the inter-physeal length in an animal of this age. This is the equivalent of a 2.5 cm regenerate in a 10-year old human.

2.5.2 Methods of Evaluation

Plain radiographs were used to assess the morphology of the regenerate and determine the alignment of the lengthened bone in 2 orthogonal (antero-posterior and medio-lateral) planes. It was important to lengthen without mal-alignment in either plane. This would influence the results produced with compressive testing adding a further confounding variable. The radiological appearances were uniform, with a fusiform regenerate and abundant new bone formation. There was no significant difference in the quality of the regenerate or the alignment seen on plain radiographs in the main experimental groups.

DXA was an available and accessible technology and using small animal software, provided a simple and reproducible method of analysis. Alternative methods of

assessment include pQCT (peripheral Quantified Computerised Tomography) *in vivo* and micro-CT *post mortem* .

Markel *et al* (Markel and Chao, 1993, Markel et al., 1991) developed a canine model to compare pQCT, SPECT (Single Photon Emission Computed Tomography) and DXA as a method of assessment of the torsional properties of healing tibial osteotomies. SPECT had the strongest association with maximum torque and torsional stiffness and pQCT and SPECT with indentation stiffness but these were not significantly different from DXA.

The principal advantage of pQCT and micro-CT over DXA relates to the resolution and accuracy of assessment of the three dimensional structure of bone.

(Hudelmaier et al., 2004) pQCT has a field view of 5-15cm with a resolution of 100-1000 μm and allows assessment of peripheral sites in intact experimental animals and determination of BMD with assessment of trabecular architecture parameters. Micro-CT has a field of view between 1-5cm with a resolution of 10-100 μm and allows assessment of complete trabecular architecture in small explanted samples or small animal peripheral sites *in vivo*. DXA does not account for the spatial distribution and inherent material properties of the tissues and pQCT would have offered a potentially superior method of assessment and should be considered as a method of assessment in future work.

DXA measurement was confined to a single assessment for each tibia and was performed by an experienced radiographer (JB) who was blinded to the treatment group. Inter and intra-rater reproducibility was not assessed and this introduced a potential bias, which could have influenced the results and their interpretation.

The lack of structural definition produced by DXA was overcome in part, by estimating the volumetric bone mineral density of the regenerate. It was not possible to make any assumptions about the degree of cortication of the regenerate, which would have added a potentially useful clinical dimension to this work. The lunar DPX software allowed an estimation of the spatial distribution of bone mineralisation by modelling the region of interest. This assumed that the regenerate was cylindrical and that mineralisation was homogenous.

There is no consistently reported method of mechanical evaluation of intact bone in general and the rabbit tibia following osteotomy and lengthening in particular. Tensile testing tests depend on the original geometry of the bone, which is highly variable, even within a single species and age group, analysis is also influenced by the integrity of the bone-clamp interface. The advantage of such tests however, is the absence of specimen machining, which is difficult to control and duplicate.

Bending tests involve complex internal stress fields and produce results that are influenced by the testing skill and orientation of the specimen and 3 and 4-point bending tests present technical difficulties associated with sample positioning. The location and size of the regenerate in this study varied between specimens and the differences in relative positions were likely to produce large and unquantifiable errors. Testing would therefore be difficult to standardise and the results difficult to interpret. Furthermore, this type of testing, when applied to fractures or experimental osteotomies, have a tendency to combine the properties of the bone with those of the developing callus in an unpredictable manner also making analysis of the regenerate bone difficult.

Torsional testing is a useful method of analysis for intact bone but was not appropriate for analysis of regenerate bone due to the mode of failure. Progressive bending of regenerate under load is a common mode of failure observed in clinical practice. This is simulated by axial compression, which was considered most representative of the *in vivo* mode of failure. Previous studies (An et al., 1994, Ohyama et al., 1994) have demonstrated a relationship between structural and mechanical properties of bone with this type of compression testing and a simple uni-axial loading test provided a reproducible method of analysis for this experiment.

The experiment aimed to recreate loading characteristics of normal walking and a rate of loading of 2 mm/min was selected to reproduce a slow strain rate consistent with previous studies (Burr et al., 1996, Lanyon et al., 1975, Rubin and Lanyon, 1982).

The dimensions of each lengthened bone were measured and used to produce a stress/strain curve for each sample after simple uni-axial compression loading to

failure. In the majority, there was a standard pattern of failure with an initial linear area, definable yield point and definable end point. A more physiological approach would involve non destructive cyclical loading as described in previous sections (Ohshima et al., 1994) and this is relevant to future work.

Data derived from DXA and uni-axial compression testing was evaluated to determine the effect of cis-platinum and adriamycin on the structural and material properties of regenerate bone. Consideration was also given to pooling the control data for the neo-adjuvant and adjuvant groups to produce a single control group to simplify the presentation of results and allow direct comparison of active groups. As with the laboratory data discussed in previous paragraphs, preliminary statistical analysis demonstrated significant differences between the control data for DXA and mechanical parameters. This suggests that the temporal relationship between anaesthesia, infusion and surgery have an independent influence on the properties of the regenerate and confirmed that a meticulously conducted and properly controlled experiment was necessary. Individual data pairs were therefore considered for each parameter.

4.5.3 The Effect of Neo-adjuvant Chemotherapy

The regenerate produced was of equivalent length, which would be expected given that this was determined by the external fixator. There was a reduction in the area of the regenerate of 0.2 cm^2 which did not reach statistical significance ($p = 0.06$). The power of this observation was 47% and 35 pairs would be required to identify a significant difference with 80% probability.

There was no obvious difference in the volume of regenerate but this should be interpreted cautiously as the power of this observation was 34% and 46 pairs would be required to give this observation appropriate power.

There were significant differences noted in bone mineral content, bone mineral density and volumetric bone mineral density with reduction in the chemotherapy group. The observations were appropriately powerful (BMC 78%, BMD 99.9%, vBMD 92%) and the observation was likely to be a real effect. This contradicts previous work (Ehrhart et al., 2002) that did not demonstrate an alteration in DXA parameters following cis-platinum infusion. A possible explanation is that the

addition of adriamycin has produced a more profound effect on regenerate mineralisation, but more detailed comment is not possible from the data produced.

The alteration in bone mineralisation was not associated with any alteration in the mechanical properties of the regenerate in this group with no significant differences in the means of any of the parameters that were tested. There was a minor reduction in the modulus of elasticity from 5.47 to 5.15×10^7 , which would be expected, as modulus/stiffness is dependant on the degree of mineralisation. The power of this observation was 4.7%, indicating that 1900 pairs would be required to give this observation a power of 80%. This is not a practical undertaking and suggests that the effect of chemotherapy is small and would be insignificant in clinical terms.

The lack of effect on the structural properties of regenerate is in agreement with Gravel *et al.*, (Gravel et al., 2003a) even with the addition of cis-platinum.

The mechanism in which adriamycin and cis-platinum affect regenerate mineralisation is not clear. The formation of regenerate bone involves a sequence of events including neo-angiogenesis (Rowe et al., 1999), chondrocyte differentiation with expression of bone matrix proteins (Sato et al., 1998), and collagen I formation (Yasui et al., 1997).

Adriamycin causes structural alterations in normal bone with reduction in cortical thickness and histological changes within the physeal plate and marrow hypoplasia (Young et al., 1975). In addition, it specifically inhibits prolyl hydroxylation leading to loss of stability of procollagen. (Sasaki et al., 1987)

cis-Platinum causes histomorphometric changes in bone including decreased volume of mineralised bone volume and decreased proportion of osteoblast covered bone (Ehrhart et al., 2002).

It was not possible to comment on the exact mechanism of action that led to these differences and future work will require histological analysis of regenerate at intervals following osteotomy.

4.5.4 The Effect of Adjuvant Chemotherapy

There was no statistically significant difference in the regenerate length, which was expected, given that this was under the control of the fixator. There was no statistically significant difference in the mean regenerate area or volume.

There was no observed difference in the bone mineral concentration, bone mineral density or volumetric bone mineral density. Power analysis in each instance indicated that 500 pairs would be required to identify a significant difference with 80% probability.

There were statistically significant reductions in energy at yield ($p = 0.01$, power 94%) and yield strain ($p = 0.01$, power 95%) in the chemotherapy group. There was no significant difference in modulus of elasticity, yield stress, energy at failure, failure stress or failure strain. This contradicts previous work (Gravel et al., 2003a) but this involved adriamycin in isolation, and the effect of cis-platinum is likely to have an additional effect.

It is not possible to comment on the lack of the observed differences in the DXA assessment on the basis of the data produced. The power of these observations was low and *post hoc* analysis indicated that large sample sizes would be required suggests that even if there is a real effect, this is negligible and unlikely to be of clinical significance. The differences in mechanical parameters is unlikely to represent a Type I error even with the relatively small sample size. The lack of correlation between the structural and mechanical properties may simply be a function of the relative insensitivity of the methods of assessment used.

Future work will require more sophisticated assessment using pQCT or micro CT to investigate changes in mineral distribution, tetracycline labelling to investigate the effect on the kinetics of bone turnover and histological assessment of the ultrastructural changes. Biochemical analysis of collagen and collagen metabolites should also be included in future experiments to investigate whether abnormalities in synthesis are a factor that may explain some of the observed differences in mineralisation. In addition the group sizes proved to be barely adequate to allow robust statistical analysis of the mechanical parameters and future work should

focus on a single specific area with larger number of animal pairs to allow improved statistical analysis.

4.6 Clinical Relevance

The purpose of this work was to explore the null hypothesis that cyclical cytotoxic chemotherapy has a no effect on the structural and mechanical properties of regenerate bone in a small mammal model of limb lengthening. The clinical relevance was to investigate the feasibility of reconstruction of segmental defects following malignant bone tumour excision by distraction osteogenesis in the presence of cytotoxic chemotherapy. The main issue is whether observed differences matter and are of sufficient concern to prevent reconstruction by this method.

The experimental model demonstrated that it is possible to perform distraction osteogenesis in this species without significant complications. The initial difficulties with wound healing were overcome by modifying the surgical technique and were not seen in the chemotherapy arm of the experiment. Pin site infection, which would be expected in this circumstance, was not seen in any case. There was a significant reduction in percentage weight gain and haemoglobin in animals that received chemotherapy but there was no effect on the peripheral leucocyte count.

Patients receiving cytotoxic drugs for malignant bone tumours frequently experience profound leucopenia and the absence of this effect may be due to the superficial nature of the animal model and an inadequate total dose of adriamycin and cis-platinum. The dose response experiment demonstrated that an increase in dose of either agent led to renal failure, which would have invalidated the model. Future work should therefore involve an increased number of cycles of chemotherapy with an increase in the total dose of these agents.

In group 1 there were statistically significant differences in DXA parameters with a difference in means of 33% (BMC), 23.3% (BMD) and 10.8% (vBMD), suggesting that neo-adjuvant chemotherapy has a detrimental effect on mineralisation.

In group 2 there was no observed difference in bone mineralisation which concurs with previous work by Erhart *et al.* (Ehrhart et al., 2002) The power of these

observations was however low and this may represent a Type II error. The high power of the equivalent observations in the neo-adjuvant group is suggestive of a real effect and the lack of difference between groups may be spurious.

There were significant differences in energy at yield and yield strain with difference in means of 30.3% (energy at yield) and 28.8% (yield strain) and appropriate statistical power. This is suggestive of a real effect and may have clinical implications, particularly as regenerate failure following fixator removal is an important consideration in human practice. The reduction in energy at yield following cytotoxic chemotherapy may be clinically important and if translated into human practice may represent an increased risk of regenerate failure following limb reconstruction.

4.7 Conclusions

It has been possible to develop an animal model that fulfilled the primary requirements of this experiment. The animals were suitable for sequential cannulation and prolonged cytotoxic infusion. There was a significant reduction in weight gain in the treated group with no evidence of clinically significant hepatic or renal failure. This model is suitable for future experiments investigating the effect of cyclical cytotoxic chemotherapy in the juvenile New Zealand White Rabbit.

It has been possible to develop a reproducible model of limb lengthening in this species. The risk of wound and pin site infection is low, bone is consistently formed in the distraction gap and the overall alignment is satisfactory in all lengthened limbs

This model is suitable for future experiments investigating limb lengthening in the juvenile New Zealand White rabbit.

There was a significant reduction in bone mineral content, bone mineral density and volumetric bone mineral density in the regenerate formed after 2 cycles of neo-adjuvant chemotherapy. This difference was not observed when adjuvant chemotherapy was given prior to and 4-days following osteotomy. It not possible to explain this effect from the data produced in this experiment and factors including the effect on neo-angiogenesis, chondrocyte differentiation, expression of bone matrix proteins and collagen I formation should be considered in future studies.

There were significant reductions in the energy at yield and yield strain after 2 cycles of neoadjuvant chemotherapy. This may have implications for the use of distraction osteogenesis in reconstruction following tumour excision. This may require either prolonged fixator wear or external support following fixator removal. It is not possible to make any more robust conclusions from the data produced by this thesis.

4.8 Future Work

The surgical model of limb lengthening using a juvenile NZW Rabbit is reproducible and has proved suitable for use in subsequent experiments. (Little et al., 2001a, Little et al., 2001b, Little et al., 2003, Uglow et al., 2003, Williams et al., 2001) The lack of a demonstrable effect following cytotoxic chemotherapy may be due to the simple fact that insufficient quantities of drugs were administered. Efficacy was assessed on the basis of reduction in percentage weight gain, with no other corroboration. It is not possible to increase the concentrations of the drugs, as this led to unacceptable complications, particularly acute renal failure. The obvious modification would be to increase the number of cycles and broaden the cytotoxic prescription. Histological analysis of retrieved organs, particularly kidney, liver, heart and growth plate should also be performed to confirm that the cytotoxic model is not associated with irreversible visceral damage, which would have an independent effect on the regenerate bone.

Future work should also assess leucocyte count at intervals of 24 to 48-hours to determine whether there is a transient reduction following cytotoxic infusion. This should be conducted in association with marrow biopsy to assess the effect of the cytotoxic drugs on stem cell numbers and function. Future work should also investigate the effect of the drugs on growth in a more sophisticated manner, with tetracycline double labelling to assess bone turnover.

This work has demonstrated that regenerate bone will form in the presence of cis-platinum and adriamycin. It has not however provided evidence at a level that allows direct translation to clinical practice. The group sizes proved to be inadequate to allow robust statistical analysis of the mechanical parameters and future work should focus on a single specific area with larger number of animal pairs to allow improved statistical power.

Assessment of the material properties of the regenerate was unsophisticated and DXA does not account for the spatial distribution and inherent material properties of the tissues. pQCT would have offered a potentially superior method of assessment and should be considered as a method of assessment in future work.

Bibliography

- ALMAN, B. A., DE BARI, A. & KRAJBICH, J. I. (1995)
Massive allografts in the treatment of osteosarcoma and Ewing sarcoma in children and adolescents.
J.Bone Joint Surg.Am, 77, 54-64.
- AN, H. S., XU, R., LIM, T. H., MCGRADY, L. & WILSON, C. (1994)
Prediction of bone graft strength using dual-energy radiographic absorptiometry.
Spine, 19, 2358-2362.
- AN, Y. H., KANG, Q. & FRIEDMAN, R. J. (1996)
Mechanical symmetry of rabbit bones studied by bending and indentation testing.
Am J Vet Res, 57, 1786-9.
- APPLETON T, G., CONNER J, W., HALL J, R. & PRENZLER P, D. (1989)
NMR Study of the reactions of the cis-diamminedichloroplatinum (II) cation with glutathione and amino acids containing a thiol group.
Inorg Chem.
- ARCAMONE, F., CASSINELLI, G., FANTINI, G., GREIN, A., OREZZI & P. (1969)
Adriamycin, 14-hydroxydaunomycin, a new antitumor antibiotic from *S. peucetius* var. *caesius*.
Biotechnol Bioeng., 11, 1110-1119.
- ARNOLDA, L., MCGRATH, B., COCKS, M., SUMITHRAN, E. & JOHNSTON, C. (1985)
Adriamycin cardiomyopathy in the rabbit: an animal model of low output cardiac failure with activation of vasoconstrictor mechanisms.
Cardiovasc.Res., 19, 378-382.
- ARONSON, J., HARRISON, B. H., STEWART, C. L. & HARP, J. H., JR. (1989)
The histology of distraction osteogenesis using different external fixators.
Clin Orthop Relat Res, 106-16.
- AYERS, R. A., MILLER, M. R., SIMSKE, S. J. & NORRDIN, R. W. (1996)
Correlation of flexural structural properties with bone physical properties: a four species survey.
Biomed Sci Instrum, 32, 251-60.
- BACCI, G., DONATI, D., MANFRINI, M., FORNI, C., BERTONI, F., GHERLINZONI, F., BIAGINI, R. & CAMPANACCI, M. (1998a)
Local recurrence after surgical or surgical-chemotherapeutic treatment of osteosarcoma of the limbs. Incidence, risk factors and prognosis.
Minerva Chir, 53, 619-629.
- BACCI, G., FERRARI, S., MERCURI, M., BERTONI, F., PICCI, P., MANFRINI, M., GASBARRINI, A., FORNI, C., CESARI, M. & CAMPANACCI, M. (1998b)
Predictive factors for local recurrence in osteosarcoma: 540 patients with extremity tumors followed for minimum 2.5 years after neoadjuvant chemotherapy.
Acta Orthop.Scand., 69, 230-236.
- BERTAZZOLI, C., SALA, L., BALLERINI, L., WATANABE, T. & FOLKERS, K. (1976)
Effect of adriamycin on the activity of the succinate dehydrogenase-coenzyme Q10 reductase of the rabbit myocardium.
Res.Commun.Chem.Pathol.Pharmacol., 15, 797-800.
- BILLINGHAM, M. E., MASON, J. W., BRISTOW, M. R. & DANIELS, J. R. (1978) Anthracycline cardiomyopathy monitored by morphologic changes.
Cancer Treat.Rep., 62, 865-872.
- BLACK, J., PERDIGON, P., BROWN, N. & POLLACK, S. R. (1984)
Stiffness and strength of fracture callus. Relative rates of mechanical maturation as evaluated by a uniaxial tensile test.
Clin Orthop Relat Res, 278-88.

- BOCHERENS-GADIENT, S. A., QUAST, U., NUSSBERGER, J., BRUNNER, H. R. & HOF, R. P. (1992)
Chronic adriamycin treatment and its effect on the cardiac beta-adrenergic system in the rabbit.
J. Cardiovasc. Pharmacol., 19, 770-778.
- BRAMWELL, V. H., BURGERS, M., SNEATH, R., SOUHAMI, R., VAN OOSTEROM, A. T., VOUTE, P. A., ROUESSE, J., SPOONER, D., CRAFT, A. W. & SOMERS, R. (1992)
A comparison of two short intensive adjuvant chemotherapy regimens in operable osteosarcoma of limbs in children and young adults: the first study of the European Osteosarcoma Intergroup.
J. Clin. Oncol., 10, 1579-1591.
- BRISTOW, M. R., MINOBE, W. A., BILLINGHAM, M. E., MARMOR, J. B., JOHNSON, G. A., ISHIMOTO, B. M., SAGEMAN, W. S. & DANIELS, J. R. (1981)
Anthracycline-associated cardiac and renal damage in rabbits. Evidence for mediation by vasoactive substances.
Lab Invest, 45, 157-168.
- BURCHARDT, H., GLOWCZEWSKIE, F. P., JR. & ENNEKING, W. F. (1983)
The effect of Adriamycin and methotrexate on the repair of segmental cortical autografts in dogs.
J Bone Joint Surg Am, 65, 103-8.
- BURR, D. B., MILGROM, C., FYHRIE, D., FORWOOD, M., NYSKA, M., FINESTONE, A., HOSHAW, S., SAIAG, E. & SIMKIN, A. (1996)
In vivo measurement of human tibial strains during vigorous activity.
Bone, 18, 405-10.
- BURROWS, H. J., WILSON, J. N. & SCALES, J. T. (1975)
Excision of tumours of humerus and femur, with restoration by internal prostheses.
J. Bone Joint Surg. Br., 57, 148-159.
- BURSTEIN, A. H. & FRANKEL, V. H. (1971)
A standard test for laboratory animal bone.
J Biomech., 4, 155-158.
- CALDERONE, A., DE CHAMPLAIN, J. & ROULEAU, J. L. (1991)
Adriamycin-induced changes to the myocardial beta-adrenergic system in the rabbit.
J. Mol. Cell Cardiol., 23, 333-342.
- CANADELL, J., FORRIOL, F. & CARA, J. A. (1994)
Removal of metaphyseal bone tumours with preservation of the epiphysis. Physeal distraction before excision.
J Bone Joint Surg Br., 76, 127-132.
- CANADELL, J., SAN-JULIAN, M., CARA, J. & FORRIOL, F. (1998)
External fixation in tumour pathology.
Int Orthop, 22, 126-30.
- CARA, J., FORRIOL, F. & CANADELL, J. (1993)
Limb Lengthening after conservative oncological surgery.
Journal of Pediatric Orthopaedics B 2, 57-61.
- CARTER, L. C., AGUIRRE, A., BOYD, B. & DELACURE, M. D. (1999)
Primary leiomyosarcoma of the mandible in a 7-year-old girl: report of a case and review of the literature.
Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 87, 477-84.
- CATAGNI, M. A., GUERRESCHI, F., LOVISETTI, L. & SINGHANIA, A. K. (2003)
Use of the Ilizarov technique to improve limb function following hemipelvectomy. A case report.
Chir Organi Mov, 88, 171-8.
- CATTANEO, R., CATAGNI, M. & JOHNSON, E. E. (1992)
The treatment of infected nonunions and segmental defects of the tibia by the methods of Ilizarov.
Clin. Orthop. Relat Res., 143-152.

- CHABNER, B. A., MYERS, C. E., COLEMAN, C. N. & JOHNS, D. G. (1975)
The clinical pharmacology of antineoplastic agents (second of two parts).
N.Engl.J Med., 292, 1159-1168.
- CHOTEL, F., BRAILLON, P., SAILHAN, F., GADEYNE, S., GELLON, J. O., PANCZER, G., PEDRINI, C. & BERARD, J. (2008)
Bone stiffness in children: part II. Objectives criteria for children to assess healing during leg lengthening.
J Pediatr Orthop, 28, 538-43.
- COLLIER, J. P., COLLIGAN, G. A. & BROWN, S. A. (1976)
Bone ingrowth into dynamically loaded porous-coated intramedullary nails.
J Biomed Mater Res, 10, 485-92.
- COURVOISIER, A., SAILHAN, F., MARY, P. & DAMSIN, J. P. (2009)
Case reports: lengthening of a vascularized free fibular graft.
Clin Orthop Relat Res, 467, 1377-84.
- CUSACK, B. J., YOUNG, S. P., DRISKELL, J. & OLSON, R. D. (1993)
Doxorubicin and doxorubicinol pharmacokinetics and tissue concentrations following bolus injection and continuous infusion of doxorubicin in the rabbit.
Cancer Chemother.Pharmacol., 32, 53-58.
- CUSACK, B. J., YOUNG, S. P., LOSEKE, V. L., HURTY, M. R., BEALS, L. & OLSON, R. D. (1992)
Effect of a low-protein diet on doxorubicin pharmacokinetics in the rabbit.
Cancer Chemother.Pharmacol., 30, 145-148.
- DAHLIN, D. C. & COVENTRY, M. B. (1967)
Osteogenic sarcoma. A study of six hundred cases.
J.Bone Joint Surg.Am., 49, 101-110.
- DE BASTIANI, G., ALDEGHERI, R., RENZI-BRIVIO, L. & TRIVELLA, G. (1987)
Limb lengthening by callus distraction (callotaxis).
J.Pediatr.Orthop., 7, 129-134.
- DOBYAN, D. C., LEVI, J., JACOBS, C., KOSEK, J. & WEINER, M. W. (1980)
Mechanism of cis-platinum nephrotoxicity: II. Morphologic observations.
J Pharmacol.Exp.Ther., 213, 551-556.
- DODD, D. A., ATKINSON, J. B., OLSON, R. D., BUCK, S., CUSACK, B. J., FLEISCHER, S. & BOUCEK, R. J., JR. (1993)
Doxorubicin cardiomyopathy is associated with a decrease in calcium release channel of the sarcoplasmic reticulum in a chronic rabbit model.
J.Clin.Invest, 91, 1697-1705.
- DONATI, D., DI LIDDO, M., ZAVATTA, M., MANFRINI, M., BACCI, G., PICCI, P., CAPANNA, R. & MERCURI, M. (2000)
Massive bone allograft reconstruction in high-grade osteosarcoma.
Clin.Orthop.Relat Res., 186-194.
- ECKARDT, J. J., SAFRAN, M. R., EILBER, F. R., ROSEN, G. & KABO, J. M. (1993)
Expandable endoprosthetic reconstruction of the skeletally immature after malignant bone tumor resection.
Clin.Orthop.Relat Res., 188-202.
- EHRHART, N., EURELL, J. A., TOMMASINI, M., CONSTABLE, P. D., JOHNSON, A. L. & FERETTI, A. (2002)
Effect of cisplatin on bone transport osteogenesis in dogs.
Am.J.Vet.Res., 63, 703-711.

- EILBER, F., GIULIANO, A., ECKARDT, J., PATTERSON, K., MOSELEY, S. & GOODNIGHT, J. (1987)
Adjuvant chemotherapy for osteosarcoma: a randomized prospective trial.
J.Clin.Oncol., 5, 21-26.
- ENNEKING, W. F. & MINDELL, E. R. (1991)
Observations on massive retrieved human allografts.
J.Bone Joint Surg.Am, 73, 1123-1142.
- ERALP, L., TOKER, B., AKGUL, T., OZGER, H., KOCAOGLU, M. & HAYAT, S. (2009)
Applications of external fixation for management of complications associated with musculoskeletal tumors and related surgery.
Acta Orthop Traumatol Turc, 43, 219-28.
- ETTINGER, L. J., DOUGLASS, H. O., JR., HIGBY, D. J., MINDELL, E. R., NIME, F., GHORAH, J. & FREEMAN, A. I. (1981)
Adjuvant adriamycin and cis-diamminedichloroplatinum (cis-platinum) in primary osteosarcoma.
Cancer, 47, 248-254.
- EVANS, F. G. & LEBOW, M. (1951)
Regional differences in some of the physical properties of the human femur.
J Appl Physiol, 3, 563-72.
- EVANS, F. G. & LEBOW, M. (1952) The strength of human compact bone as revealed by engineering technics.
Am J Surg, 83, 326-31.
- EYRES, K. S., BELL, M. J. & KANIS, J. A. (1993a)
Methods of assessing new bone formation during limb lengthening. Ultrasonography, dual energy X-ray absorptiometry and radiography compared.
J.Bone Joint Surg.Br., 75, 358-364.
- EYRES, K. S., BELL, M. J. & KANIS, J. A. (1993b)
New bone formation during leg lengthening. Evaluated by dual energy X-ray absorptiometry.
J.Bone Joint Surg.Br., 75, 96-106.
- FRANK, J. A., CHOYKE, P. L., GIRTON, M. E., AUSTIN, H. A., SIEVENPIPER, C., INSCOE, S. W., BLACK, J. L., CARVLIN, M. J. & DWYER, A. J. (1989)
Gadolinium-DTPA enhanced dynamic MR imaging in the evaluation of cisplatin nephrotoxicity.
J Comput Assist Tomogr, 13, 448-59.
- FYDA, T. M., CALLAGHAN, J. J., FULGHUM, C. S., SEABER, A. V. & MYERS, B. S. (1995)
A model of cortical window healing in the rabbit.
Orthopedics, 18, 177-84.
- GEBHARDT, M. C., FLUGSTAD, D. I., SPRINGFIELD, D. S. & MANKIN, H. J. (1991)
The use of bone allografts for limb salvage in high-grade extremity osteosarcoma.
Clin.Orthop.Relat Res., 181-196.
- GLASSER, D. B. & LANE, J. M. (1991)
Stage IIB osteogenic sarcoma.
Clin Orthop Relat Res, 29-39.
- GONZALES-VITALE, J. C., HAYES, D. M., CVITKOVIC, E. & STERNBERG, S. S. (1977)
The renal pathology in clinical trials of cis-platinum (II) diamminedichloride.
Cancer, 39, 1362-1371.
- GONZALEZ-HERRANZ, P., BURGOS-FLORES, J., OCETE-GUZMAN, J. G., LOPEZ-MONDEJAR, J. A. & AMAYA, S. (1995)
The management of limb-length discrepancies in children after treatment of osteosarcoma and Ewing's sarcoma.
J Pediatr Orthop, 15, 561-5.

- GRAVEL, C. A., LE, T. T. & CHAPMAN, M. W. (2003)
Effect of neoadjuvant chemotherapy on distraction osteogenesis in the goat model.
Clin Orthop Relat Res, 213-24.
- GREEN, S. A., JACKSON, J. M., WALL, D. M., MARINOW, H. & ISHKANIAN, J. (1992)
Management of segmental defects by the Ilizarov intercalary bone transport method.
Clin.Orthop.Relat Res., 136-142.
- GRIFFON, D. J., WALLACE, L. J. & BECHTOLD, J. E. (1995)
Biomechanical properties of canine corticocancellous bone frozen in normal saline solution.
Am J Vet.Res., 56, 822-825.
- GRIMER, R. J., CARTER, S. R. & PYNSENT, P. B. (1997)
The cost-effectiveness of limb salvage for bone tumours.
J.Bone Joint Surg.Br., 79, 558-561.
- GUICHET, J. M., BRAILLON, P., BODENREIDER, O. & LASCOMBES, P. (1998)
Periosteum and bone marrow in bone lengthening: a DEXA quantitative evaluation in rabbits.
Acta Orthop Scand, 69, 527-31.
- GUPTA, A., POLLOCK, R., CANNON, S. R., BRIGGS, T. W., SKINNER, J. & BLUNN, G. (2006)
A knee-sparing distal femoral endoprosthesis using hydroxyapatite-coated extracortical plates.
Preliminary results.
J.Bone Joint Surg.Br., 88, 1367-1372.
- HAHN, S. B., KIM, S. H., CHO, N. H., CHOI, C. J., KIM, B. S. & KANG, H. J. (2007)
Treatment of osteofibrous dysplasia and associated lesions.
Yonsei Med J, 48, 502-10.
- HAMANISHI, C., KAWABATA, T., YOSHII, T. & TANAKA, S. (1995)
Bone mineral density changes in distracted callus stimulated by pulsed direct electrical current.
Clin.Orthop.Relat Res., 247-252.
- HANAFUSA, S., MATSUSUE, Y., YASUNAGA, T., YAMAMURO, T., OKA, M., SHIKINAMI, Y. & IKADA, Y. (1995)
Biodegradable plate fixation of rabbit femoral shaft osteotomies. A comparative study.
Clin Orthop Relat Res, 262-71.
- HANDA, K. & SATO, S. (1975)
Generation of free radicals of quinone group-containing anti-cancer chemicals in NADPH-microsome system as evidenced by initiation of sulfite oxidation.
Gann, 66, 43-47.
- HANLON, M. & KRAJBICH, J. I. (1999)
Rotationplasty in skeletally immature patients. Long-term followup results.
Clin.Orthop.Relat Res., 75-82.
- HARDER, H. C. & ROSENBERG, B. (1970)
Inhibitory effects of anti-tumor platinum compounds on DNA, RNA and protein syntheses in mammalian cells in vitro.
Int.J Cancer, 6, 207-216.
- HAYES, D. M., CVITKOVIC, E., GOLBEY, R. B., SCHEINER, E., HELSON, L. & KRAKOFF, I. H. (1977)
High dose cis-platinum diammine dichloride: amelioration of renal toxicity by mannitol diuresis.
Cancer, 39, 1372-1381.
- HILLMANN, A., HOFFMANN, C., GOSHEGER, G., KRAKAU, H. & WINKELMANN, W. (1999)
Malignant tumor of the distal part of the femur or the proximal part of the tibia: endoprosthetic replacement or rotationplasty. Functional outcome and quality-of-life measurements.
J.Bone Joint Surg.Am, 81, 462-468.

- HSU, R. W., WOOD, M. B., SIM, F. H. & CHAO, E. Y. (1997)
Free vascularised fibular grafting for reconstruction after tumour resection.
J.Bone Joint Surg.Br., 79, 36-42.
- HUDELMAIER, M., KUHN, V., LOCHMULLER, E. M., WELL, H., PRIEMEL, M., LINK, T. M. & ECKSTEIN, F. (2004)
Can geometry-based parameters from pQCT and material parameters from quantitative ultrasound (QUS) improve the prediction of radial bone strength over that by bone mass (DXA)?
Osteoporos Int, 15, 375-81.
- HULSEN, K. K. (1896)
The specific gravity, resilience and strength of bony tissue.
Bull. Lab. Biol. St. Petersburg, 1, 7 -35.
- IACOBELLIS, C. & OLMEDA, A. (2004)
The Ilizarov method in the treatment of malignant neoplasms of the tibia.
Chir Organi Mov, 89, 245-50.
- ILIZAROV, G. A. (1989a)
The tension-stress effect on the genesis and growth of tissues: Part II. The influence of the rate and frequency of distraction.
Clin.Orthop.Relat Res., 263-285.
- ILIZAROV, G. A. (1989b)
The tension-stress effect on the genesis and growth of tissues. Part I. The influence of stability of fixation and soft-tissue preservation.
Clin.Orthop.Relat Res., 249-281.
- ILIZAROV, G. A. (1990)
Clinical application of the tension-stress effect for limb lengthening.
Clin.Orthop.Relat Res., 8-26.
- ILIZAROV, G. A. & LEDYAEV, V. I. (1992)
The replacement of long tubular bone defects by lengthening distraction osteotomy of one of the fragments. 1969.
Clin.Orthop.Relat Res., 7-10.
- ILIZAROV, S., BLYAKHER, A. & ROZBRUCH, S. R. (2007)
Lengthening of a free fibular graft after sarcoma resection of the humerus.
Clin Orthop Relat Res, 457, 242-6.
- JACOBS, P. A. (1984)
Limb salvage and rotationplasty for osteosarcoma in children.
Clin.Orthop.Relat Res., 217-222.
- JAENKE, R. S. (1976)
Delayed and progressive myocardial lesions after adriamycin administration in the rabbit.
Cancer Res., 36, 2958-2966.
- JAENKE, R. S., DEPREZ-DECAMPENEERE, D. & TROUET, A. (1980)
Cardiotoxicity and comparative pharmacokinetics of six anthracyclines in the rabbit.
Cancer Res., 40, 3530-3536.
- JAFFE, N., FREI, E., III, TRAGGIS, D. & BISHOP, Y. (1974)
Adjuvant methotrexate and citrovorum-factor treatment of osteogenic sarcoma.
N.Engl.J.Med., 291, 994-997.
- JARKA, D. E., NICHOLAS, R. W. & ARONSON, J. (1998)
Effect of methotrexate on distraction osteogenesis.
Clin Orthop Relat Res, 209-15.

- JAVADPOUR, N. (1985)
Pharmacology and clinical applications of cis-platinum.
Urology, 25, 155-160.
- JONES, B. R., BHALLA, R. B., MLADEK, J., KALEYA, R. N., GRALLA, R. J., ALCOCK, N. W., SCHWARTZ, M. K., YOUNG, C. W. & REIDENBERG, M. M. (1980)
Comparison of methods of evaluating nephrotoxicity of cis-platinum.
Clin.Pharmacol.Ther., 27, 557-562.
- JONES, S. M., KIRBY, M. S., HARDING, S. E., VESCOVA, G., WANLESS, R. B., DALLA LIBERA, L. D. & POOLE-WILSON, P. A. (1990)
Adriamycin cardiomyopathy in the rabbit: alterations in contractile proteins and myocyte function.
Cardiovasc.Res., 24, 834-842.
- KABUKCUOGLU, Y., GRIMER, R. J., TILLMAN, R. M. & CARTER, S. R. (1999)
Endoprosthetic replacement for primary malignant tumors of the proximal femur.
Clin.Orthop.Relat Res., 8-14.
- KAPUKAYA, A., SUBASI, M., KANDIYA, E., OZATES, M. & YILMAZ, F. (2000)
Limb reconstruction with the callus distraction method after bone tumor resection.
Arch Orthop Trauma Surg, 120, 215-8.
- KAWEBLUM, M., AGUILAR, M. C., BLANCAS, E., KAWEBLUM, J., LEHMAN, W. B., GRANT, A. D. & STRONGWATER, A. M. (1994)
Histological and radiographic determination of the age of physeal closure of the distal femur, proximal tibia, and proximal fibula of the New Zealand white rabbit.
J.Orthop.Res., 12, 747-749.
- KEIZER, H. G., PINEDO, H. M., SCHUURHUIS, G. J. & JOENJE, H. (1990)
Doxorubicin (adriamycin): a critical review of free radical-dependent mechanisms of cytotoxicity.
Pharmacol.Ther., 47, 219-231.
- KOCIBA, R. J. & SLEIGHT, S. D. (1971)
Acute toxicologic and pathologic effects of cis-diamminedichloroplatinum (NSC-119875) in the male rat.
Cancer Chemother.Rep., 55, 1-8.
- KOJIMOTO, H., YASUI, N., GOTO, T., MATSUDA, S. & SHIMOMURA, Y. (1988)
Bone lengthening in rabbits by callus distraction. The role of periosteum and endosteum.
J Bone Joint Surg Br, 70, 543-9.
- KOTZ, R. & SALZER, M. (1982)
Rotation-plasty for childhood osteosarcoma of the distal part of the femur.
J.Bone Joint Surg.Am, 64, 959-969.
- KRAUS, H. (1968)
On the Mechanical Properties and Behavior of Human Compact Bone.
Biom. Eng. Med. Phys.
- LAFFARGUE, P., HILDEBRAND, H. F., RTAIMATE, M., FRAYSSINET, P., AMOUREUX, J. P. & MARCHANDISE, X. (1999)
Evaluation of human recombinant bone morphogenetic protein-2-loaded tricalcium phosphate implants in rabbits' bone defects.
Bone, 25, 55S-58S.
- LAITINEN, M., HARDES, J., AHRENS, H., GEBERT, C., LEIDINGER, B., LANGER, M., WINKELMANN, W. & GOSHEGER, G. (2005)
Treatment of primary malignant bone tumours of the distal tibia.
Int Orthop, 29, 255-9.
- LAMMENS, J. & FABRY, G. (1992)
Reconstruction of bony defects using the Ilizarov "bone transport" technique. A preliminary report.
Arch Orthop Trauma Surg, 111, 70-2.

- LANYON, L. E., HAMPSON, W. G., GOODSHIP, A. E. & SHAH, J. S. (1975)
Bone deformation recorded in vivo from strain gauges attached to the human tibial shaft.
Acta Orthop Scand, 46, 256-68.
- LAZARUS, M. L., ROSSNER, K. L. & ANDERSON, K. M. (1980)
Adriamycin-induced alterations of the action potential in rat papillary muscle.
Cardiovasc.Res., 14, 446-450.
- LEE, J. W. (1996)
Mechanical strength of regenerate bones treated with cytotoxic chemotherapy.
University of Sydney.
- LEE, R. S., WEITZEL, S., EASTWOOD, D. M., MONSELL, F., PRINGLE, J., CANNON, S. R. & BRIGGS, T. W. (2006)
Osteofibrous dysplasia of the tibia: Is there a need for a radical approach?
J Bone Joint Surg Br., 88, 658-664.
- LENAZ, L. & PAGE, J. A. (1976)
Cardiotoxicity of adriamycin and related anthracyclines.
Cancer Treat.Rev., 3, 111-120.
- LENOBLE, E., LEWERTOWSKI, J. M. & GOUTALLIER, D. (1995)
Reconstruction of compound tibial and soft tissue loss using a traction histogenesis technique.
J Trauma, 39, 356-60.
- LEXER, E. (1925)
Joint transplantation and arthroplasty.
Surg.Gynecol.Obstet., 40, 782-809.
- LINK, M. P., GOORIN, A. M., HOROWITZ, M., MEYER, W. H., BELASCO, J., BAKER, A., AYALA, A. & SHUSTER, J. (1991)
Adjuvant chemotherapy of high-grade osteosarcoma of the extremity. Updated results of the Multi-Institutional Osteosarcoma Study.
Clin.Orthop.Relat Res., 8-14.
- LINK, M. P., GOORIN, A. M., MISER, A. W., GREEN, A. A., PRATT, C. B., BELASCO, J. B., PRITCHARD, J., MALPAS, J. S., BAKER, A. R. & KIRKPATRICK, J. A. (1986)
The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity.
N.Engl.J.Med., 314, 1600-1606.
- LIPPMAN, A. J., HELSON, C., HELSON, L. & KRAKOFF, I. H. (1973)
Clinical trials of cis-diamminedichloroplatinum (NSC-119875).
Cancer Chemother.Rep., 57, 191-200.
- LITTLE, D. G., CORNELL, M. S., BRIODY, J., COWELL, C. T., ARBUCKLE, S. & COOKE-YARBOROUGH, C. M. (2001a)
Intravenous pamidronate reduces osteoporosis and improves formation of the regenerate during distraction osteogenesis. A study in immature rabbits.
J Bone Joint Surg Br, 83, 1069-74.
- LITTLE, D. G., CORNELL, M. S., HILE, M. S., BRIODY, J., COWELL, C. T. & BILSTON, L. (2001b)
Effect of pamidronate on distraction osteogenesis and fixator-related osteoporosis.
Injury, 32 Suppl 4, SD14-20.
- LITTLE, D. G., SMITH, N. C., WILLIAMS, P. R., BRIODY, J. N., BILSTON, L. E., SMITH, E. J., GARDINER, E. M. & COWELL, C. T. (2003)
Zoledronic acid prevents osteopenia and increases bone strength in a rabbit model of distraction osteogenesis.
J Bone Miner Res, 18, 1300-7.

- MAFFULLI, N. C., J. C. SHER, A. LAM, T. P. (1997)
Dual-energy X-ray absorptiometry predicts bone formation in lower limb callotasis lengthening.
Ann R Coll Surg Engl 79, 250-256.
- MALAWER, M., BUCH, R., REAMAN, G., PRIEBAT, D., POTTER, B., KHURANA, J., SHMOOKLER, B., PATTERSON, K. & SCHULOF, R. (1991)
Impact of two cycles of preoperative chemotherapy with intraarterial cisplatin and intravenous doxorubicin on the choice of surgical procedure for high-grade bone sarcomas of the extremities.
Clin. Orthop. Relat Res., 214-222.
- MANKIN, H. J., GEBHARDT, M. C. & TOMFORD, W. W. (1987)
The use of frozen cadaveric allografts in the management of patients with bone tumors of the extremities.
Orthop. Clin. North Am, 18, 275-289.
- MARCOVE, R. C. (1978)
En bloc resections for osteogenic sarcoma.
Cancer Treat. Rep., 62, 225-231.
- MARKEL, M. D. & CHAO, E. Y. (1993)
Noninvasive monitoring techniques for quantitative description of callus mineral content and mechanical properties.
Clin. Orthop., 37-45.
- MARKEL, M. D., WIKENHEISER, M. A., MORIN, R. L., LEWALLEN, D. G. & CHAO, E. Y. (1991)
The determination of bone fracture properties by dual-energy X-ray absorptiometry and single-photon absorptiometry: a comparative study.
Calcif. Tissue Int., 48, 392-399.
- MCELHANEY, J. H. & BYARS, E. F. (1965)
Dynamic Response of Biological Materials.
ASME Paper No. 65-WA-HUF-9, 1-8.
- MCKENNA, R. J., SCHWINN, C. P., SOONG, K. Y. & HIGINBOTHAM, N. L. (1966)
Sarcomata of the osteogenic series (osteosarcoma, fibrosarcoma, parosteal osteogenic sarcoma and sarcoma arising in abnormal bone.
IN MCKENNA RJ, S. C. P. S. K. Y. (Ed.).
- MIDDLEMAN, E., LUCE, J. & FREI, E., III (1971)
Clinical trials with adriamycin.
Cancer, 28, 844-850.
- MILLETT, P. J., LANE, J. M. & PALETTA, G. A., JR. (2000)
Limb salvage using distraction osteogenesis.
Am J Orthop, 29, 628-32.
- MINOW, R. A., BENJAMIN, R. S., LEE, E. T. & GOTTLIEB, J. A. (1977)
Adriamycin cardiomyopathy--risk factors.
Cancer, 39, 1397-1402.
- MITLAK, B. H., RODDA, C. P., VON DECK, M. D., DOBROLET, N. C., NEER, R. M. & NUSSBAUM, S. R. (1991)
Pamidronate reduces PTH-mediated bone loss in a gene transfer model of hyperparathyroidism in rats.
J. Bone Miner. Res., 6, 1317-1321.
- MIWA, N., KANAIDE, H., MENO, H. & NAKAMURA, M. (1986)
Adriamycin and altered membrane functions in rat hearts.
Br. J. Exp. Pathol., 67, 747-755.

- MUNCHAUSEN, L. L. & RAHN, R. O. (1975)
Biologic and chemical effects of cis-dichlorodiammineplatinum (II) (NSC-119875) on DNA.
Cancer Chemother.Rep., 59, 643-646.
- NAGGAR, L., CHEVALLEY, F., BLANC, C. H. & LIVIO, J. J. (1993)
Treatment of large bone defects with the Ilizarov technique.
J Trauma, 34, 390-393.
- NAJJAR, T. A. & SAAD, S. Y. (2001)
Cisplatin pharmacokinetics and its nephrotoxicity in diabetic rabbits.
Chemotherapy, 47, 128-35.
- OH, C. W., SHETTY, G. M., SONG, H. R., KYUNG, H. S., OH, J. K., MIN, W. K., LEE, B. W. & PARK, B. C. (2008)
Submuscular plating after distraction osteogenesis in children.
J Pediatr Orthop B, 17, 265-9.
- OHYAMA, M., MIYASAKA, Y., SAKURAI, M., YOKOBORI, A. T., JR. & SASAKI, S. (1994)
The mechanical behavior and morphological structure of callus in experimental callotasis.
Biomed Mater Eng, 4, 273-81.
- OZAKI, T., NAKATSUKA, Y., KAWAI, A., AKAZAWA, H., KUNISADA, T. & INOUE, H. (1998)
Reconstruction of bone defect using the bone transport technique for a case of osteosarcoma of the femur.
Acta Med.Okayama, 52, 67-70.
- PAAVOLAINEN, P. (1978)
Studies on mechanical strength of bone. I. Torsional strength of normal rabbit tibio-fibular bone.
Acta Orthop Scand, 49, 497-505.
- PALEY, D. (2002)
Principles of deformity correction,
Berlin, New York, Springer.
- PALEY, D., CATAGNI, M. A., ARGNANI, F., VILLA, A., BENEDETTI, G. B. & CATTANEO, R. (1989)
Ilizarov treatment of tibial nonunions with bone loss.
Clin.Orthop.Relat Res., 146-165.
- PALEY, D. & MAAR, D. C. (2000)
Ilizarov bone transport treatment for tibial defects.
J Orthop.Trauma, 14, 76-85.
- PERSING, J. A., MORGAN, E. P., CRONIN, A. J. & WOLCOTT, W. P. (1991)
Skull base expansion: craniofacial effects.
Plast.Reconstr.Surg., 87, 1028-1033.
- PEYRONE, M. (1845)
über die Einwirkung des Ammoniaks auf PlatinchlorÄr.
Ann Chem Pharm 1845; 51: 1-29.
- PILLA, A. A., MONT, M. A., NASSER, P. R., KHAN, S. A., FIGUEIREDO, M., KAUFMAN, J. J. & SIFFERT, R. S. (1990)
Non-invasive low-intensity pulsed ultrasound accelerates bone healing in the rabbit.
J.Orthop.Trauma, 4, 246-253.
- PREVOT, J., PONCELET, T., LEMELLE, J. L., LASCOMBES, P., BLANQUART, D., MEMBRE, H. & OLIVE, D. (1988)
Study of distraction osteogenesis in an animal body submitted to anticancer chemotherapy.
Chir Pediatr, 29, 226-30.

- PROKUSKI, L. J. & MARSH, J. L. (1994)
Segmental bone deficiency after acute trauma. The role of bone transport.
Orthop.Clin.North Am, 25, 753-763.
- RAUBER, A. A. (1876)
Elasticitat und festigkeit der knochen.
Leipzeig, Engelmann.
- REICHEL, H., LEBEK, S., ALTER, C. & HEIN, W. (1998)
Biomechanical and densitometric bone properties after callus distraction in sheep.
Clin Orthop Relat Res, 237-46.
- ROBERTS, P., CHAN, D., GRIMER, R. J., SNEATH, R. S. & SCALES, J. T. (1991)
Prosthetic replacement of the distal femur for primary bone tumours.
J.Bone Joint Surg.Br., 73, 762-769.
- ROE, S. C., PIJANOWSKI, G. J. & JOHNSON, A. L. (1988)
Biomechanical properties of canine cortical bone allografts: effects of preparation and storage.
Am J Vet.Res., 49, 873-877.
- ROGERS, M. J., JACKSON, M., LIVINGSTONE, J. A., MONSELL, F. P. & ATKINS, R. M. (2007)
Bone Transport Using a Stacked Spatial Frame.
J Bone Joint Surg Br 88-B, 431.
- ROMANOS, E., GHANEM, I., DAGHER, F. & KHARRAT, K. (2008)
Is bone lengthening over an intramedullary prosthesis stem possible and safe?
J Pediatr Orthop B, 17, 307-9.
- ROSEN, G. (1985)
Preoperative (neoadjuvant) chemotherapy for osteogenic sarcoma: a ten year experience.
Orthopedics, 8, 659-664.
- ROSEN, G., MARCOVE, R. C., HUVOS, A. G., CAPARROS, B. I., LANE, J. M., NIRENBERG, A., CACAVIO, A. & GROSHEN, S. (1983)
Primary osteogenic sarcoma: eight-year experience with adjuvant chemotherapy.
J.Cancer Res.Clin.Oncol., 106 Suppl, 55-67.
- ROSEN, G., MURPHY, M. L., HUVOS, A. G., GUTIERREZ, M. & MARCOVE, R. C. (1976)
Chemotherapy, en bloc resection, and prosthetic bone replacement in the treatment of osteogenic sarcoma.
Cancer, 37, 1-11.
- ROSENBERG, B., VANCAMP, L. & KRIGAS, T. (1965)
Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode.
Nature, 205, 698-699.
- ROWE, N. M., MEHRARA, B. J., LUCHS, J. S., DUDZIAK, M. E., STEINBRECH, D. S., ILLEI, P. B., FERNANDEZ, G. J., GITTES, G. K. & LONGAKER, M. T. (1999)
Angiogenesis during mandibular distraction osteogenesis.
Ann Plast Surg, 42, 470-5.
- RUBIN, C. T. & LANYON, L. E. (1982)
Limb mechanics as a function of speed and gait: a study of functional strains in the radius and tibia of horse and dog.
J Exp Biol, 101, 187-211.
- SAID, G. Z. & EL SHERIF, E. K. (1995)
Resection-shortening-distraction for malignant bone tumours. A report of two cases.
J Bone Joint Surg Br., 77, 185-188.
- SALZER-KUNTSCHIK, M., BRAND, G. & DELLING, G. (1983) Determination of the degree of morphological regression following chemotherapy in malignant bone tumors.
Pathologe, 4, 135-41.

- SASAKI, T., HOLEYFIELD, K. C. & UITTO, J. (1987)
Doxorubicin-induced inhibition of prolyl hydroxylation during collagen biosynthesis in human skin fibroblast cultures. Relevance to impaired wound healing.
J Clin Invest, 80, 1735-1741.
- SATO, M., YASUI, N., NAKASE, T., KAWAHATA, H., SUGIMOTO, M., HIROTA, S., KITAMURA, Y., NOMURA, S. & OCHI, T. (1998)
Expression of bone matrix proteins mRNA during distraction osteogenesis.
J Bone Miner Res, 13, 1221-31.
- SCHIFFER, J., WALACH, N., LUSHKOV, G., NYSKA, A., GUR, R. & POLLAK, L. (1996)
Effect of the combination of cis-platinum and radiation in the rabbit's brain.
Neurol Res, 18, 454-6.
- SCHILLER, C., WINDHAGER, R., FELLINGER, E. J., SALZER-KUNTSCHIK, M., KAIDER, A. & KOTZ, R. (1995)
Extendable tumour endoprotheses for the leg in children.
J Bone Joint Surg.Br., 77, 608-614.
- SCHINDLER, O. S., CANNON, S. R., BRIGGS, T. W. & BLUNN, G. W. (1997)
Stanmore custom-made extendible distal femoral replacements. Clinical experience in children with primary malignant bone tumours.
J Bone Joint Surg.Br., 79, 927-937.
- SEDDON, H. J. & SCALES, J. T. (1949)
A polythene substitute for the upper two-thirds of the shaft of the femur.
Lancet, 2, 795.
- SEDLIN, E. D. & HIRSCH, C. (1966)
Factors affecting the determination of the physical properties of femoral cortical bone.
Acta Orthop Scand, 37, 29-48.
- SHALABY, S., SHALABY, H. & BASSIONY, A. (2006)
Limb salvage for osteosarcoma of the distal tibia with resection arthrodesis, autogenous fibular graft and Ilizarov external fixator.
J Bone Joint Surg Br, 88, 1642-6.
- SHEA, K. G., COLEMAN, D. A., SCOTT, S. M., COLEMAN, S. S. & CHRISTIANSON, M. (1997)
Microvascularized free fibular grafts for reconstruction of skeletal defects after tumor resection.
J.Pediatr.Orthop., 17, 424-432.
- SIMON, M. A., ASCHLIMAN, M. A., THOMAS, N. & MANKIN, H. J. (1986)
Limb-salvage treatment versus amputation for osteosarcoma of the distal end of the femur.
J Bone Joint Surg.Am, 68, 1331-1337.
- SINGAL, P. K. & PIERCE, G. N. (1986)
Adriamycin stimulates low-affinity Ca²⁺ binding and lipid peroxidation but depresses myocardial function.
Am J Physiol, 250, H419-H425.
- SINHA, B. K., KATKI, A. G., BATIST, G., COWAN, K. H. & MYERS, C. E. (1987)
Differential formation of hydroxyl radicals by adriamycin in sensitive and resistant MCF-7 human breast tumor cells: implications for the mechanism of action.
Biochemistry, 26, 3776-3781.
- SONG, H. R., CHO, S. H., KOO, K. H., JEONG, S. T., PARK, Y. J. & KO, J. H. (1998)
Tibial bone defects treated by internal bone transport using the Ilizarov method.
Int.Orthop., 22, 293-297.

- SOUHAMI, R. L., CRAFT, A. W., VAN DER EIJKEN, J. W., NOOIJ, M., SPOONER, D., BRAMWELL, V. H., WIERZBICKI, R., MALCOLM, A. J., KIRKPATRICK, A., USCINSKA, B. M., VAN GLABBEKE, M. & MACHIN, D. (1997)
Randomised trial of two regimens of chemotherapy in operable osteosarcoma: a study of the European Osteosarcoma Intergroup.
Lancet, 350, 911-917.
- SPRINGFIELD, D. S., SCHMIDT, R., GRAHAM-POLE, J., MARCUS, R. B., JR., SPANIER, S. S. & ENNEKING, W. F. (1988)
Surgical treatment for osteosarcoma.
J.Bone Joint Surg.Am, 70, 1124-1130.
- STILLER, C. A., BIELACK, S. S., JUNDT, G. & STELIAROVA-FOUCHER, E. (2006)
Bone tumours in European children and adolescents, 1978-1997. Report from the Automated Childhood Cancer Information System project.
Eur J Cancer, 42, 2124-35.
- SWEETNAM, R. (1973)
Amputation in osteosarcoma. Disarticulation of the hip or high thigh amputation for lower femoral growths?
J.Bone Joint Surg.Br., 55, 189-192.
- TAYLOR, G. I., MILLER, G. D. & HAM, F. J. (1975)
The free vascularized bone graft. A clinical extension of microvascular techniques.
Plast.Reconstr.Surg., 55, 533-544.
- TERJESEN, T. & BENUM, P. (1983)
Stress-protection after external fixation on the intact rabbit tibia.
Acta Orthop Scand, 54, 648-54.
- TEWEY, K. M., ROWE, T. C., YANG, L., HALLIGAN, B. D. & LIU, L. F. (1984)
Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II.
Science, 226, 466-468.
- TORBERT, J. T., FOX, E. J., HOSALKAR, H. S., OGILVIE, C. M. & LACKMAN, R. D. (2005)
Endoprosthetic reconstructions: results of long-term followup of 139 patients.
Clin.Orthop.Relat Res., 438, 51-59.
- TRITTON, T. R. (1991)
Cell surface actions of adriamycin.
Pharmacol.Ther., 49, 293-309.
- TSUCHIYA, H., ABDEL-WANIS, M. E., SAKURAKICHI, K., YAMASHIRO, T. & TOMITA, K. (2002)
Osteosarcoma around the knee. Intraepiphyseal excision and biological reconstruction with distraction osteogenesis.
J.Bone Joint Surg.Br., 84, 1162-1166.
- TSUCHIYA, H., TOMITA, K., MINEMATSU, K., MORI, Y., ASADA, N. & KITANO, S. (1997)
Limb salvage using distraction osteogenesis. A classification of the technique.
J.Bone Joint Surg.Br., 79, 403-411.
- UGLOW, M. G., PEAT, R. A., HILE, M. S., BILSTON, L. E., SMITH, E. J. & LITTLE, D. G. (2003)
Low-intensity ultrasound stimulation in distraction osteogenesis in rabbits.
Clin Orthop Relat Res, 303-12.
- VAN NES, C. P. (1950)
Rotationplasty for congenital defects of the femur.
J.Bone Joint Surg.Br., 32 B 12-16.

- VON HOFF, D. D., LAYARD, M. W., BASA, P., DAVIS, H. L., JR., VON HOFF, A. L., ROZENCWEIG, M. & MUGGIA, F. M. (1979)
Risk factors for doxorubicin-induced congestive heart failure.
Ann.Intern.Med., 91, 710-717.
- WALSH, W. R., HAMDY, R. C. & EHRLICH, M. G. (1994)
Biomechanical and physical properties of lengthened bone in a canine model.
Clin Orthop Relat Res, 230-8.
- WANG, J. J., CORTES, E., SINKS, L. F. & HOLLAND, J. F. (1971)
Therapeutic effect and toxicity of adriamycin in patients with neoplastic disease.
Cancer, 28, 837-843.
- WANLESS, R. B., ANAND, I. S., POOLE-WILSON, P. A. & HARRIS, P. (1987)
An experimental model of chronic cardiac failure using adriamycin in the rabbit: central haemodynamics and regional blood flow.
Cardiovasc.Res., 21, 7-13.
- WEILAND, A. J. (1981)
Current concepts review: vascularized free bone transplants.
J.Bone Joint Surg.Am, 63, 166-169.
- WEISS, R. B. & POSTER, D. S. (1982)
The renal toxicity of cancer chemotherapeutic agents.
Cancer Treat.Rev., 9, 37-56.
- WERTHEIM, M. G. (1847)
Memoire sur Velasticite et la cohesion des principaux tissus du corps humain.
Ann. Chim. Phys., 21, 385 - 414.
- WIERNIK, P. H. & SERPICK, A. A. (1972)
A randomized clinical trial of daunorubicin and a combination of prednisone, vincristine, 6-mercaptopurine, and methotrexate in adult acute nonlymphocytic leukemia.
Cancer Res., 32, 2023-2026.
- WILLIAMS, P. R., SMITH, N. C., COOKE-YARBOROUGH, C. & LITTLE, D. G. (2001)
Bisphosphonates and nephrocalcinosis in a rabbit leg lengthening model: a histological and therapeutic comparison.
Pharmacol Toxicol, 89, 149-52.
- WRIGHT, T. M. & HAYES, W. C. (1976)
Tensile Testing of Bone Over a Wide Range of Strain Rates: Effects of Strain Rate, Microstructure and Density.
Med. Biol. Eng., 14, 671-680.
- YASUI, N., SATO, M., OCHI, T., KIMURA, T., KAWAHATA, H., KITAMURA, Y. & NOMURA, S. (1997)
Three modes of ossification during distraction osteogenesis in the rat.
J Bone Joint Surg Br., 79, 824-830.
- YOUNG, D. M., FIORAVANTI, J. L., OLSON, H. M. & PRIEUR, D. J. (1975)
Chemical and morphologic alterations of rabbit bone induced by adriamycin.
Calcif. Tissue Res., 18, 47-63.
- ZAHRINGER, J. (1981)
The regulation of protein synthesis in heart muscle under normal conditions and in the adriamycin-cardiomyopathy.
Klin.Wochenschr., 59, 1273-1287.

Appendix 1

WESTERN SYDNEY AREA HEALTH SERVICE ANIMAL CARE & ETHICS COMMITTEE

*Westmead Hospital
Westmead 2145
Telephone (02) 845 6176
Facsimile (02) 845 7626*

ANIMAL RESEARCH AUTHORITY CERTIFICATE

F. Monsell
Orthopaedics Department, New Childrens Hospital

is authorised by the

*WESTERN SYDNEY AREA HEALTH SERVICE
ANIMAL CARE AND ETHICS COMMITTEE*

to perform animal research in the Project

Effect of chemotherapy on bone mineralisation.

Protocol 765-11-96

only in accordance with the conditions
of the approval of the project.

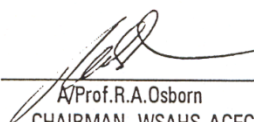
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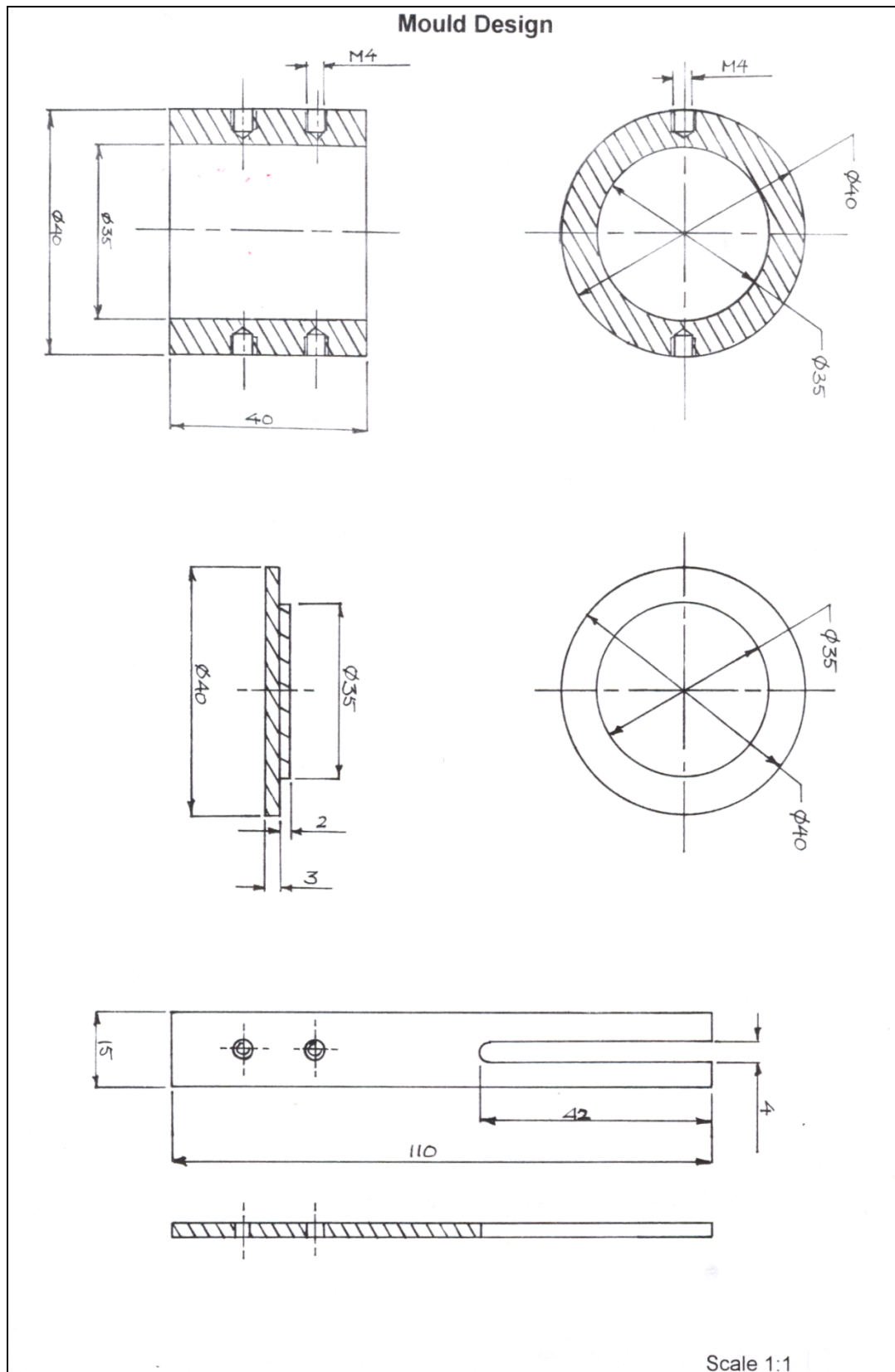
Chief Executive Officer

Dr. O.G. Curteis



Prof. R.A. Osborn
CHAIRMAN, WSAHS ACEC
Issued Dec., 1995

Appendix 2



Appendix 3

Abbreviations

AP	Antero-posterior
ASAMI	Association for the Study and Application of the Methods of Ilizarov
BA	Bone area
BMC	Bone mineral content
BMD	Bone mineral density
vBMD	Volumetric Bone Mineral Density
BUN	Blood Urea Nitrogen
COSS	Cooperative Osteosarcoma Study
CO	Cardiac output
CPK	Creatinine phosphokinase
CULAS	Combined Universities Laboratory Animal Services
DXA	Dual Energy X-Ray Absorptiometry
DNA	Deoxyribonucleic Acid
E	Modulus of elasticity
Energy F	Energy to failure
Energy Y	Energy to yield
ESR	Erythrocyte Sedimentation Rate
EOI	European Osteosarcoma Intergroup
FDA	American Food and Drug Administration
FM	Fergal Monsell
F Strain	Failure strain
F Stress	Failure stress
FBC	Full Blood Count
γGT	Gamma-Glutamyl Transpeptidase
G-CSF	Granulocyte Colony Stimulating Factor
HR	Heart rate
JWL	Jay Wong Lee
Lat	Lateral
LC	Lyndal Cohen
LDH	Lactate Dehydrogenase

LRS	Limb Reconstruction System
MIOS	Multi-Institutional Osteosarcoma Study
MTSS	Musculoskeletal Tumour Society Score
MRI	Magnetic Resonance Imaging
NSW	New South Wales
NZW	New Zealand White
PCT	Proximal convoluted tubule
PMMA	Poly Methyl Methacrylate
p-QCT	Peripheral Quantified Computerised Tomography
RNA	Ribonucleic Acid
RNOH	Royal National Orthopaedic Hospital
ROI	Region of Interest
SF 36	Short Form 36
SPECT	Single Photon Emission Computed Tomography
SPF	Specific Pathogen Free
TJ	Troy James
TPR	Total peripheral resistance
TSF	Taylor Spatial Frame
Y Strain	Yield strain
Y Stress	Yield stress

Appendix 4

Neo-adjuvant Group Regenerate Data

Rabbit	1	3	4	6	7	9	10	12	13	14
Length	0.84	0.6	0.72	0.84	0.84	0.96	0.72	0.48	0.84	0.6
Area	0.67	0.9	0.51	1.19	1.32	0.5	0.84	0.66	0.49	0.62
Volume	0.56	0.54	0.37	1.00	1.11	0.48	0.6	0.31	0.41	0.37
Oblique Plane Alignment	6.00	0.00	5.66	0.00	2.00	11.66	2.83	2.00	0.00	6.00

Rabbit	DD	G	H	I	J	K	M	N	O	Q
Length	0.84	0.72	0.84	0.84	0.84	0.60	0.6	0.60	0.72	0.72
Area	0.48	0.55	0.62	0.62	0.48	0.53	0.56	0.65	0.56	0.62
Volume	0.41	0.40	0.52	0.52	0.41	0.32	0.33	0.39	0.40	0.45
Oblique Plane Alignment	8.49	11.66	4.47	7.21	4.47	6.00	7.21	0.00	4.47	4.47

Adjuvant Group Regenerate Data

Rabbit	15	16	17	18	20	21	22	23	24	25
Length	0.60	0.72	0.72	0.60	0.48	0.60	0.84	0.84	0.72	0.48
Area	0.47	0.61	0.71	0.43	0.33	0.43	0.61	0.77	0.57	0.46
Volume	0.29	0.4	0.54	0.24	0.18	0.24	0.34	0.55	0.36	0.33
Oblique Plane Alignment	2.94	4.47	6.43	6.32	6.32	4.47	6.32	8.94	6.49	4.55

Rabbit	AA	BB	CC	R	T	U	V	W	X	Y	Z
Length	0.72	0.72	0.72	0.72	0.72	0.72	0.60	0.60	0.84	0.72	0.84
Area	0.55	0.41	0.45	0.51	0.64	0.63	0.64	0.62	0.15	0.37	0.45
Volume	0.40	0.30	0.33	0.37	0.46	0.45	0.38	0.37	0.12	0.27	0.38
Oblique Plane Alignment	2.00	2.00	4.47	4.47	4.47	8.94	4.47	2.00	5.66	4.00	7.21

Neo-adjuvant Group DXA Data

Rabbit	1	3	4	6	7	9	10	12	13	14
LAT BMD	5.11E-01	4.84E-01	3.54E-01	6.77E-01	6.39E-01	5.18E-01	4.87E-01	5.43E-01	5.04E-01	5.70E-01
LAT BMC	4.42E-01	2.93E-01	2.09E-01	5.32E-01	6.54E-01	3.61E-01	3.82E-01	2.07E-01	3.08E-01	2.67E-01
LAT Area	8.64E-01	6.05E-01	5.90E-01	7.87E-01	1.02E+00	6.98E-01	7.85E-01	3.82E-01	6.12E-01	4.68E-01
LAT Width	1.03E+00	1.01E+00	8.19E-01	1.09E+00	1.22E+00	7.27E-01	1.09E+00	7.95E-01	7.29E-01	7.80E-01
AP BMD	6.06E-01	4.14E-01	3.81E-01	4.55E-01	5.53E-01	4.30E-01	5.87E-01	3.81E-01	4.18E-01	4.35E-01
AP BMC	4.23E-01	2.83E-01	2.17E-01	5.31E-01	6.41E-01	3.63E-01	4.14E-01	1.92E-01	3.00E-01	2.66E-01
AP Area	6.98E-01	6.84E-01	5.69E-01	1.17E+00	1.16E+00	8.43E-01	7.05E-01	5.04E-01	7.18E-01	6.12E-01
AP Width	8.31E-01	1.14E+00	7.90E-01	1.39E+00	1.38E+00	8.78E-01	9.79E-01	1.05E+00	8.55E-01	1.02E+00
AREA	6.72E-01	9.03E-01	5.08E-01	1.19E+00	1.32E+00	5.01E-01	8.38E-01	6.56E-01	4.90E-01	6.25E-01
VOL	5.64E-01	5.42E-01	3.66E-01	1.00E+00	1.11E+00	4.81E-01	6.03E-01	3.15E-01	4.11E-01	3.75E-01
LAT VBMD	7.83E-01	5.41E-01	5.71E-01	5.31E-01	5.90E-01	7.50E-01	6.33E-01	6.58E-01	7.49E-01	7.12E-01
AP VBMD	7.50E-01	5.23E-01	5.93E-01	5.30E-01	5.79E-01	7.54E-01	6.86E-01	6.10E-01	7.30E-01	7.09E-01

Rabbit	G	H	I	J	K	M	N	O	Q	DD
LAT BMD	4.34E-01	3.49E-01	3.88E-01	3.28E-01	3.35E-01	3.96E-01	3.42E-01	4.10E-01	4.68E-01	3.33E-01
LAT BMC	2.47E-01	2.69E-01	2.93E-01	2.26E-01	1.64E-01	2.01E-01	1.80E-01	2.57E-01	2.80E-01	2.30E-01
LAT Area	5.69E-01	7.70E-01	7.56E-01	6.89E-01	4.90E-01	5.08E-01	5.26E-01	6.27E-01	5.98E-01	6.91E-01
LAT Width	7.90E-01	9.17E-01	9.00E-01	8.20E-01	8.16E-01	8.47E-01	8.76E-01	8.72E-01	8.30E-01	8.23E-01
AP BMD	3.74E-01	3.53E-01	4.08E-01	3.57E-01	3.37E-01	4.13E-01	3.38E-01	4.59E-01	4.28E-01	3.77E-01
AP BMC	2.40E-01	2.54E-01	3.02E-01	2.26E-01	1.68E-01	2.08E-01	1.92E-01	2.71E-01	2.94E-01	2.36E-01
AP Area	6.41E-01	7.18E-01	7.42E-01	6.33E-01	4.97E-01	5.03E-01	5.69E-01	5.90E-01	6.87E-01	6.26E-01
AP Width	8.90E-01	8.55E-01	8.83E-01	7.53E-01	8.29E-01	8.38E-01	9.48E-01	8.20E-01	9.54E-01	7.46E-01
AREA	5.52E-01	6.16E-01	6.24E-01	4.85E-01	5.31E-01	5.57E-01	6.52E-01	5.62E-01	6.22E-01	4.82E-01
VOL	3.98E-01	5.17E-01	5.24E-01	4.07E-01	3.19E-01	3.34E-01	3.91E-01	4.04E-01	4.48E-01	4.05E-01
LAT VBMD	6.21E-01	5.20E-01	5.59E-01	5.55E-01	5.14E-01	6.01E-01	4.60E-01	6.36E-01	6.25E-01	5.68E-01
AP VBMD	6.04E-01	4.91E-01	5.76E-01	5.55E-01	5.27E-01	6.22E-01	4.91E-01	6.70E-01	6.57E-01	5.83E-01

Adjuvant Group DXA Data

Rabbit	15	16	17	18	20	21	22	23	24	25
LAT BMD	3.90E-01	4.14E-01	4.61E-01	3.84E-01	3.51E-01	3.78E-01	4.16E-01	4.39E-01	3.09E-01	6.14E-01
LAT BMC	1.74E-01	2.62E-01	3.35E-01	1.71E-01	1.16E-01	1.60E-01	2.55E-01	3.48E-01	1.85E-01	2.30E-01
LAT Area	4.46E-01	6.34E-01	7.27E-01	4.46E-01	3.31E-01	4.25E-01	6.12E-01	7.92E-01	5.98E-01	3.74E-01
LAT Width	7.44E-01	8.80E-01	1.01E+00	7.44E-01	6.90E-01	7.08E-01	7.29E-01	9.43E-01	8.30E-01	7.80E-01
AP BMD	3.28E-01	4.22E-01	5.05E-01	3.67E-01	3.30E-01	3.17E-01	4.23E-01	4.45E-01	3.60E-01	3.99E-01
AP BMC	1.60E-01	2.46E-01	3.45E-01	1.53E-01	1.07E-01	1.35E-01	2.53E-01	3.30E-01	1.97E-01	2.15E-01
AP Area	4.90E-01	5.83E-01	6.84E-01	4.18E-01	3.24E-01	4.25E-01	5.98E-01	7.42E-01	5.47E-01	5.40E-01
AP Width	8.16E-01	8.10E-01	9.50E-01	6.96E-01	6.75E-01	7.08E-01	7.11E-01	8.83E-01	7.60E-01	1.13E+00
AREA	4.77E-01	5.60E-01	7.54E-01	4.07E-01	3.66E-01	3.94E-01	4.07E-01	6.54E-01	4.95E-01	6.89E-01
VOL	2.86E-01	4.03E-01	5.43E-01	2.44E-01	1.76E-01	2.36E-01	3.42E-01	5.49E-01	3.57E-01	3.31E-01
LAT VBMD	6.08E-01	6.50E-01	6.17E-01	7.01E-01	6.61E-01	6.77E-01	7.46E-01	6.33E-01	5.19E-01	6.95E-01
AP VBMD	5.59E-01	6.10E-01	6.36E-01	6.27E-01	6.09E-01	5.72E-01	7.40E-01	6.01E-01	5.52E-01	6.50E-01

Rabbit	AA	BB	CC	R	T	U	V	W	X	Y	Z
LAT BMD	4.28E-01	4.98E-01	3.67E-01	3.85E-01	5.10E-01	4.30E-01	3.62E-01	4.11E-01	2.98E-01	4.07E-01	4.76E-01
LAT BMC	2.59E-01	2.15E-01	1.80E-01	2.38E-01	3.23E-01	3.07E-01	2.16E-01	2.16E-01	1.05E-01	1.93E-01	2.92E-01
LAT Area	6.05E-01	4.33E-01	4.91E-01	6.19E-01	6.34E-01	7.13E-01	5.98E-01	5.26E-01	3.53E-01	4.75E-01	6.12E-01
LAT Width	8.40E-01	6.01E-01	6.81E-01	8.60E-01	8.80E-01	9.90E-01	9.96E-01	8.76E-01	4.20E-01	6.60E-01	7.29E-01
AP BMD	4.20E-01	3.30E-01	2.79E-01	4.16E-01	4.65E-01	5.11E-01	4.57E-01	3.84E-01	2.76E-01	3.38E-01	4.33E-01
AP BMC	2.54E-01	2.06E-01	1.69E-01	2.28E-01	3.10E-01	2.98E-01	2.24E-01	2.07E-01	1.03E-01	1.73E-01	2.84E-01
AP Area	6.05E-01	6.26E-01	6.07E-01	5.48E-01	6.66E-01	5.83E-01	4.90E-01	5.40E-01	3.74E-01	5.12E-01	6.55E-01
AP Width	8.40E-01	8.70E-01	8.43E-01	7.61E-01	9.25E-01	8.10E-01	8.16E-01	9.00E-01	4.46E-01	7.11E-01	7.80E-01
AREA	5.54E-01	4.11E-01	4.51E-01	5.14E-01	6.39E-01	6.30E-01	6.38E-01	6.19E-01	1.47E-01	3.69E-01	4.47E-01
VOL	3.99E-01	2.96E-01	3.25E-01	3.70E-01	4.60E-01	4.53E-01	3.83E-01	3.72E-01	1.24E-01	2.65E-01	3.75E-01
LAT VBMD	6.49E-01	7.27E-01	5.54E-01	6.43E-01	7.02E-01	6.77E-01	5.64E-01	5.81E-01	8.50E-01	7.27E-01	7.78E-01
AP VBMD	6.37E-01	6.97E-01	5.21E-01	6.16E-01	6.73E-01	6.57E-01	5.85E-01	5.57E-01	8.33E-01	6.52E-01	7.57E-01

Neo-adjuvant Group Mechanical Data

Rabbit	3	4	6	7	9	10	12	13	14
Mean E	4.50E+08	5.63E+08	3.17E+08	1.94E+08	1.44E+09	6.07E+08	5.70E+08	6.66E+08	4.83E+08
Mean eY	5.56E+07	5.19E+07	6.26E+06	3.08E+07	9.67E+06	2.17E+07	4.08E+07	2.50E+07	4.10E+07
Y Stress	1.41E+07	2.32E+07	5.64E+06	1.09E+07	1.66E+07	1.68E+07	2.05E+07	1.95E+07	1.94E+07
Y Strain	3.10E-02	4.17E-02	1.99E-02	5.83E-02	1.10E-02	4.14E-02	3.70E-02	3.75E-02	5.37E-02
Mean eF	5.03E+07	6.97E+07	3.69E+07	3.82E+07	1.75E+07	4.68E+07	6.18E+07	8.07E+07	9.14E+07
F Stress	1.66E+07	2.43E+07	8.67E+06	1.14E+07	1.95E+07	1.95E+07	2.12E+07	2.45E+07	2.23E+07
F Strain	4.75E-02	4.83E-02	6.07E-02	6.48E-02	1.50E-02	5.27E-02	4.64E-02	6.21E-02	8.48E-02
Post Yield e	-5.30E+06	1.78E+07	3.06E+07	7.40E+06	7.83E+06	2.51E+07	2.10E+07	5.57E+07	5.04E+07

Rabbit	G	H	I	J	K	M	N	O	Q	DD
Mean E	7.13E+08	5.20E+08	3.52E+08	6.85E+08	5.61E+08	5.55E+08	5.41E+08	7.55E+08	6.20E+08	6.13E+08
mean eY	2.23E+07	3.42E+07	3.07E+07	9.50E+06	2.47E+07	3.72E+07	3.57E+07	2.24E+07	5.07E+06	2.40E+07
Y Stress	1.94E+07	1.84E+07	1.39E+07	1.23E+07	1.63E+07	1.94E+07	1.94E+07	1.79E+07	9.65E+06	1.78E+07
Y Strain	2.80E-02	4.15E-02	4.11E-02	1.66E-02	3.05E-02	4.65E-02	3.67E-02	2.53E-02	1.82E-02	3.77E-02
Mean eF	6.78E+07	3.42E+07	3.52E+08	2.20E+07	4.00E+07	8.31E+07	6.14E+07	3.44E+07	7.76E+07	3.64E+07
F Stress	2.75E+07	1.84E+07	1.81E+07	1.47E+07	1.71E+07	2.10E+07	2.17E+07	2.03E+07	1.67E+07	1.91E+07
F Strain	4.69E-02	4.15E-02	6.00E-02	2.64E-02	4.23E-02	6.20E-02	4.89E-02	3.27E-02	6.97E-02	4.40E-02
Post Yield e	4.55E+07	0.00E-01	3.21E+08	1.25E+07	1.53E+07	4.59E+07	2.57E+07	1.20E+07	7.25E+07	1.24E+07

Adjuvant Group Mechanical Data

Rabbit	15	16	17	18	20	21	22	23	24	25
mean E	3.50E+08	5.38E+08	2.52E+08	4.85E+08	5.15E+08	5.72E+08	7.17E+08	3.96E+08	5.05E+08	2.89E+08
mean eY	4.19E+07	3.19E+07	5.08E+07	4.00E+07	2.85E+07	3.69E+07	3.91E+07	4.06E+07	3.78E+07	4.50E+07
Y stress	1.90E+07	1.89E+07	1.66E+07	1.82E+07	1.79E+07	2.16E+07	2.41E+07	1.68E+07	1.89E+07	1.66E+07
Y strain	5.73E-02	4.05E-02	8.36E-02	4.46E-02	4.31E-02	4.47E-02	4.13E-02	5.68E-02	4.48E-02	6.28E-02
mean eF	4.19E+07	6.54E+07	6.20E+07	8.50E+07	8.62E+07	5.19E+07	1.07E+08	4.06E+07	3.78E+07	6.27E+07
F stress	1.90E+07	2.09E+07	1.72E+07	1.90E+07	2.13E+07	2.27E+07	2.53E+07	1.68E+07	1.89E+07	1.71E+07
F strain	5.73E-02	5.83E-02	9.10E-02	6.90E-02	6.85E-02	5.23E-02	6.77E-02	5.68E-02	4.48E-02	7.32E-02
Post Yield e	0.00E-01	3.35E+07	1.12E+07	4.50E+07	5.77E+07	1.50E+07	6.79E+07	0.00E-01	0.00E-01	1.77E+07

Rabbit	AA	CC	R	T	U	V	W	Y	Z
mean E	6.15E+08	4.68E+08	2.70E+08	1.05E+09	3.81E+08	4.55E+08	2.17E+08	7.13E+08	9.17E+08
mean eY	1.72E+07	1.11E+07	1.63E+07	2.24E+07	3.62E+07	4.41E+07	2.13E+07	3.69E+07	4.10E+07
Y stress	1.46E+07	1.03E+07	9.84E+06	2.23E+07	1.61E+07	1.89E+07	9.06E+06	2.23E+07	2.76E+07
Y strain	2.60E-02	2.96E-02	3.62E-02	2.74E-02	4.75E-02	5.19E-02	4.93E-02	3.24E-02	3.08E-02
mean eF	4.94E+07	2.12E+07	9.37E+07	5.92E+07	6.54E+07	5.55E+07	3.18E+07	4.28E+07	5.36E+07
F stress	1.73E+07	1.20E+07	1.49E+07	2.71E+07	1.73E+07	1.96E+07	1.02E+07	2.28E+07	2.84E+07
F strain	4.72E-02	3.83E-02	8.77E-02	4.15E-02	6.36E-02	5.88E-02	6.13E-02	3.71E-02	3.57E-02
Post Yield e	3.22E+07	1.01E+07	7.74E+07	3.68E+07	2.92E+07	1.14E+07	1.05E+07	5.90E+06	1.26E+07